



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification<sup>5</sup> :

C12N 15/00, 5/00

A1

(11) International Publication Number:

WO 91/19796

(43) International Publication Date:

26 December 1991 (26.12.91)

(21) International Application Number: PCT/US91/04006

(22) International Filing Date: 7 June 1991 (07.06.91)

(30) Priority data:

536,397	12 June 1990 (12.06.90)	US
537,458	14 June 1990 (14.06.90)	US
597,694	17 October 1990 (17.10.90)	US

(71) Applicant: BAYLOR COLLEGE OF MEDICINE [US/US]; One Baylor Plaza, Houston, TX 77030 (US).

(72) Inventors: BRADLEY, Allan ; 5619 Wigton, Houston, TX 77096 (US). DAVIS, Ann, C. ; 5415 Braesvalley, #819, Houston, TX 77096 (US). HASTY, Paul ; 1928 North Braeswood, Houston, TX 77030 (US).

(74) Agent: AUERBACH, Jeffrey, I.; Weil, Gotshal &amp; Manges, 1615 L Street, N.W., Washington, DC 20036 (US).

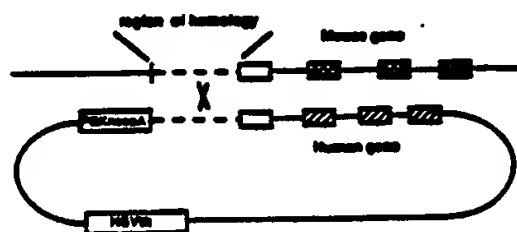
(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published

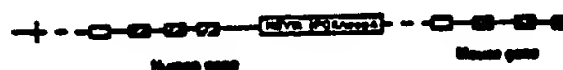
With international search report.

(54) Title: METHOD FOR HOMOLOGOUS RECOMBINATION IN ANIMAL AND PLANT CELLS

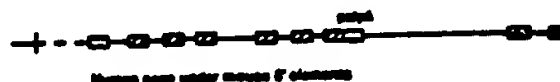
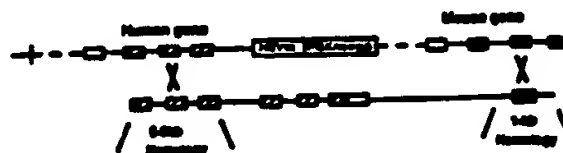
• Step1, homologous recombination: Adding the human replacement with an insertion vector.



[ ] Mouse non-coding exon  
 [ ] Human non-coding exon  
 [ ] Human coding exon  
 [ ] Mouse coding exon  
 - - - Mouse 5' flanking sequence, may include the promoter



Step2: Reconstruct function, remove duplicated promoter, add additional 3' human sequences. Select in - F1AU (100%)



(57) Abstract

A method for producing animal cells which contain a desired gene sequence which has been inserted into a predetermined gene sequence by homologous recombination. The method permits the production of animal cells which have subtle and precise modifications of gene sequence and expression.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

1  
2  
3  
4  
5  
6  
7 **TITLE OF THE INVENTION:**  
8  
9

10 Method for Homologous Recombination in Animal  
11 and Plant Cells  
12  
13

14  
15 **CROSS-REFERENCE TO RELATED APPLICATIONS:**  
16

17 This application is a continuation-in-part application  
18 of U.S. Patent Application Serial No. 07/537,458, filed on  
19 June 14, 1990.  
20

21  
22 **FIELD OF THE INVENTION:**

23 The invention is directed toward recombinant DNA  
24 technology, and more specifically, toward methods for  
25 modifying endogenous genes in a chimeric or transgenic  
26 animal or plant. The invention further pertains to the  
27 animals/plants produced through application of the method,  
28 and to the use of the method in medicine and agriculture.  
29 This invention was supported by Government funds. The  
30 Government has certain rights in this invention.  
31

32  
33 **BACKGROUND OF THE INVENTION:**

34  
35 **I. Chimeric and Transgenic Animals**

36 Recent advances in recombinant DNA and genetic  
37 technologies have made it possible to introduce and express  
38 a desired gene sequence in a recipient animal. Through the  
39 use of such methods, animals have been engineered to contain  
40

-2-

1       gen sequences that are not normally or naturally present in  
2       an unaltered animal. The techniques have also been used to  
3       produce animals which exhibit altered expression of  
4       naturally present gene sequences.

5       The animals produced through the use of these methods  
6       are known as either "chimeric" or "transgenic" animals. In  
7       a "chimeric" animal, only some of the animal's cells contain  
8       and express the introduced gene sequence, whereas other  
9       cells have been unaltered. The capacity of a chimeric  
10      animal to transmit the introduced gene sequence to its  
11      progeny depends upon whether the introduced gene sequences  
12      are present in the germ cells of the animal. Thus, only  
13      certain chimeric animals can pass along the desired gene  
14      sequence to their progeny.

15      In contrast, all of the cells of a "transgenic" animal  
16      contain the introduced gene sequence. Consequently, a  
17      transgenic animal is capable of transmitting the introduced  
18      gene sequence to its progeny.

## 19                                   II.       Production of Transgenic Animals: 20                                   Microinjection Methods 21

22                   The most widely used method through which transgenic  
23      animals have been produced involves injecting a DNA molecule  
24      into the male pronucleus of a fertilized egg (Brinster, R.L.  
25      et al., Cell 27:223 (1981); Costantini, F. et al., Nature  
26      294:92 (1981); Harbers, K. et al., Nature 293:540 (1981);  
27      Wagner, E.F. et al., Proc. Natl. Acad. Sci. (U.S.A.) 78:5016  
28      (1981); Gordon, J.W. et al., Proc. Natl. Acad. Sci. (U.S.A.)  
29      73:1260 (1976)).  
30

31      The gene sequence being introduced need not be incor-  
32      porated into any kind of self-replicating plasmid or virus  
33      (Jaenisch, R., Science, 240:1468-1474 (1988)). Indeed, the

1        presence of vector DNA has been found, in many cases, to be  
2        undesirable (Hammer, R.E. et al., Science 235:53 (1987);  
3        Chada, K. et al., Nature 319:685 (1986); Kollias, G. et al.,  
4        Cell 46:89 (1986); Shani, M., Molec. Cell. Biol. 6:2624  
5        (1986); Chada, K. et al., Nature 314:377 (1985); Townes, T.  
6        et al., EMBO J. 4:1715 (1985)).

7        After being injected into the recipient fertilized egg,  
8        the DNA molecules are believed to recombine with one another  
9        to form extended head-to-tail concatemers. It has been  
10       proposed that such concatemers occur at sites of double-  
11       stranded DNA breaks at random sites in the egg's  
12       chromosomes, and that the concatemers are inserted and  
13       integrated into such sites (Brinster, R.L. et al., Proc.  
14       Natl. Acad. Sci. (U.S.A.) 82:4438 (1985)). Although it is,  
15       thus, possible for the injected DNA molecules to be  
16       incorporated at several sites within the chromosomes of the  
17       fertilized egg, in most instances, only a single site of  
18       insertion is observed (Jaenisch, R., Science, 240:1468-1474  
19       (1988); Meade, H. et al. (U.S. Patent 4,873,316)).

20       Once the DNA molecule has been injected into the  
21       fertilized egg cell, the cell is implanted into the uterus  
22       of a recipient female, and allowed to develop into an  
23       animal. Since all of the animal's cells are derived from  
24       the implanted fertilized egg, all of the cells of the  
25       resulting animal (including the germ line cells) shall  
26       contain the introduced gene sequence. If, as occurs in  
27       about 30% of events, the first cellular division occurs  
28       before the introduced gene sequence has integrated into the  
29       cell's genome, the resulting animal will be a chimeric  
30       animal.

31       By breeding and inbreeding such animals, it has been  
32       possible to produce heterozygous and homozygous transgenic  
33       animals. Despite any unpredictability in the formation of

-4-

1 such transgenic animals, the animals have generally been  
2 found to be stable, and to be capable of producing offspring  
3 which retain and express the introduced gene sequence.

4 Since microinjection causes the injected DNA to be  
5 incorporated into the genome of the fertilized egg through  
6 a process involving the disruption and alteration of the  
7 nucleotide sequence in the chromosome of the egg at the  
8 insertion site, it has been observed to result in the  
9 alteration, disruption, or loss of function of the  
10 endogenous egg gene in which the injected DNA is inserted.  
11 Moreover, substantial alterations (deletions, duplications,  
12 rearrangements, and translocations) of the endogenous egg  
13 sequences flanking the inserted DNA have been observed  
14 (Mahon, K.A. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:1165  
15 (1988); Covarrubias, Y. et al., Proc. Natl. Acad. Sci.  
16 (U.S.A.) 83:6020 (1986); Mark, W. et al., Cold Spr. Harb.  
17 Symp. Quant. Biol. 50:453 (1985)). Indeed, lethal mutations  
18 or gross morphological abnormalities have been observed  
19 (Jaenisch, R., Science 240:1468-1474 (1988); First, N.L. et  
20 al., Amer. Meat Sci. Assn. 39th Reciprocal Meat Conf. 39:41  
21 (1986)).

22 Significantly, it has been observed that even if the  
23 desired gene sequence of the microinjected DNA molecule is  
24 one that is naturally found in the recipient egg's genome,  
25 integration of the desired gene sequence rarely occurs at  
26 the site of the natural gene (Brinster, R.L. et al., Proc.  
27 Natl. Acad. Sci. (U.S.A.) 86:7087-7091 (1989)). Moreover,  
28 introduction of the desired gene sequence does not generally  
29 alter the sequence of the originally present egg gene.

30 Although the site in the fertilized egg's genome into  
31 which the injected DNA ultimately integrates cannot be  
32 predetermined, it is possible to control the expression of  
33 the desired gene sequence such that, in the animal,

-5-

1        expression of the sequence will occur in an organ or tissue  
2        specific manner (reviewed by Westphal, H., FASEB J. 3:117  
3        (1989); Jaenisch, R., Science 240:1468-1474 (1988)).

4        The success rate for producing transgenic animals is  
5        greatest in mice. Approximately 25% of fertilized mouse  
6        eggs into which DNA has been injected, and which have been  
7        implanted in a female, will become transgenic mice. A lower  
8        rate has been thus far achieved with rabbits, sheep, cattle,  
9        and pigs (Jaenisch, R., Science 240:1468-1474 (1988);  
10        Hammer, R.E. et al., J. Animal. Sci. 63:269 (1986); Hammer,  
11        R.E. et al., Nature 315:680 (1985); Wagner, T.E. et al.,  
12        Theriogenology 21:29 (1984)). The lower rate may reflect  
13        greater familiarity with the mouse as a genetic system, or  
14        may reflect the difficulty of visualizing the male  
15        pronucleus of the fertilized eggs of many farm animals  
16        (Wagner, T.E. et al., Theriogenology 21:29 (1984)).

17        Thus, the production of transgenic animals by  
18        microinjection of DNA suffers from at least two major  
19        drawbacks. First, it can be accomplished only during the  
20        single-cell stage of an animal's life. Second, it requires  
21        the disruption of the natural sequence of the DNA, and thus  
22        is often mutagenic or teratogenic (Gridley, T. et al.,  
23        Trends Genet. 3:162 (1987)).

24        III.        Production of Chimeric and Transgenic Animals:  
25               Recombinant Viral and Retroviral Methods  
26

27        Chimeric and transgenic animals may also be produced  
28        using recombinant viral or retroviral techniques in which  
29        the gene sequence is introduced into an animal at a multi-  
30        cell stage. In such methods, the desired gene sequence is  
31        introduced into a virus or retrovirus. Cells which are  
32        infected with the virus acquire the introduced gene  
33

-6-

1 s quence. If th virus or retrovirus infects every cell of  
2 the animal, then the method r sults in th producti n of a  
3 transgenic animal. If, however, the virus infects only some  
4 of the animal's cells, then a chimeric animal is produced.

5 The general advantage of viral or retroviral methods of  
6 producing transgenic animals over those methods which  
7 involve the microinjection of non-replicating DNA, is that  
8 it is not necessary to perform the genetic manipulations at  
9 a single cell stage. Moreover, infection is a highly  
10 efficient means for introducing the DNA into a desired cell.

11 Recombinant retroviral methods for producing chimeric or  
12 transgenic animals have the advantage that retroviruses  
13 integrate into a host's genome in a precise manner,  
14 resulting generally in the presence of only a single  
15 integrated retrovirus (although multiple insertions may  
16 occur). Rearrangements of the host chromosome at the sit  
17 of integration are, in general, limited to minor deletions  
18 (Jaenisch, R., Science 240:1468-1474 (1988); see also,  
19 Varmus, H., In: RNA Tumor Viruses (Weiss, R. et al., Eds.),  
20 Cold Spring Harbor Press, Cold Spring Harbor, NY, pp. 369-  
21 512 (1982)). The method is, however, as mutagenic as micro-  
22 injection methods.

23 Chimeric animals have, for example, been produced by  
24 incorporating a desired gene sequence into a virus (such as  
25 bovine papilloma virus or polyoma) which is capable of  
26 infecting the cells of a host animal. Upon infection, the  
27 virus can be maintained in an infected cell as an  
28 extrachromosomal episome (Elbrecht, A. et al., Molec. Cell.  
29 Biol. 7:1276 (1987); Lacey, M. et al., Nature 322:609  
30 (1986); Leopold, P. et al., Cell 51:885 (1987)). Although  
31 this method decreases the mutagenic nature of  
32 chimeric/transgenic animal formation, it does so by  
33 decreasing germ line stability, and increasing oncogenicity.



1 pluripotent embryonic stem cells (referred to as "ES"  
2 c lls) are cells which may be btained from embryos until  
3 the early post-implantation stag of embryogen sis. The  
4 cells may be propagated in culture, and are able to  
5 differentiate either in vitro or in vivo upon implantation  
6 into a mouse as a tumor. ES cells have a normal karyotype  
7 (Evans, M.J. et al., Nature 292:154-156 (1981); Martin, G.R.  
8 et al., Proc. Natl. Acad. Sci. (U.S.A.) 78:7634-7638  
9 (1981)).

10 Upon injection into a blastocyst of a developing embryo,  
11 ES cells will proliferate and differentiate, thus resulting  
12 in the production of a chimeric animal. ES cells ar  
13 capable of colonizing both the somatic and germ-line  
14 lineages of such a chimeric animal (Robertson, E. et al.,  
15 Cold Spring Harb. Conf. Cell Prolif. 10:647-663 (1983);  
16 Bradley A. et al., Nature 309:255-256 (1984); Bradley, A. et  
17 al., Curr. Top. Devel. Biol. 20:357-371 (1986); Wagner, E.F.  
18 et al., Cold Spring Harb. Symp. Quant. Biol. 50:691-700  
19 (1985); (all of which references are incorporated herein by  
20 reference).

21 In this method, ES cells are cultured in vitro, and  
22 infected with a viral or retroviral vector containing th  
23 gene sequence of interest. Chimeric animals generated with  
24 retroviral vectors have been found to have germ cells which  
25 either lack the introduced gene sequence, or contain the  
26 introduced sequence but lack the capacity to produce prog ny  
27 cells capable of expressing the introduced sequence (Evans,  
28 M.J. et al., Cold Spring Harb. Symp. Quant. Biol. 50:685-689  
29 (1985); Stewart, C.L. et al., EMBO J. 4:3701-3709 (1985);  
30 Robertson, L. et al., Nature (1986); which references are  
31 incorporated herein by reference).

32 Because ES cells may be propagated in vitro, it is  
33 possibl to manipulate such cells using the techniques of

somatic cell genetics. Thus, it is possible to select ES cells which carry mutations (such as in the hprt gene (encoding hypoxanthine phosphoribosyl transferase) (Hooper, M. et al., Nature 326:292-295 (1987); Kuehn, M.R. et al., Nature 326:295-298 (1987)). Such selected cells can then be used to produce chimeric or transgenic mice which fail to express an active HPRT enzyme, and thus provide animal models for diseases (such as the Lesch-Nyhan syndrome which is characterized by an HPRT deficiency) (Doetschman, T. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:8583-8587 (1988)).

As indicated above, it is possible to generate a transgenic animal from a chimeric animal (whose germ line cells contain the introduced gene sequence) by inbreeding.

The above-described methods permit one to screen for the desired genetic alteration prior to introducing the transfected ES cells into the blastocyst. One drawback of these methods, however, is the inability to control the site or nature of the integration of the vector.

#### IV. Production of Chimeric and Transgenic Animals: Plasmid Methods

The inherent drawbacks of the above-described methods for producing chimeric and transgenic animals have caused researchers to attempt to identify additional methods through which such animals could be produced.

Gossler, A. et al., for example, have described the use of a plasmid vector which had been modified to contain the gene for neomycin phosphotransferase (nptII gene) to transfect ES cells in culture. The presence of the nptII gene conferred resistance to the antibiotic G418 to ES cells that had been infected by the plasmid (Gossler, A. et al., Proc. Natl. Acad. Sci. (U.S.A.) 83:9065-9069 (1986), which

1 r f renc is inc rp rat d h r in by r f renc ). The  
2 chimeric animals which rec ived th plasmid and which became  
3 resistant to G418, were found to have integrated the vector  
4 into their chromosomes. Takahashi, Y. et al. have  
5 described the use of a plasmid to produce chimeric mice  
6 cells which expressed an avian crystallin gene (Development  
7 102:258-269 (1988), incorporated herein by reference). Th  
8 avian gene was incorporated into a plasmid which contained  
9 the nptII gene. Resulting chimeric animals were found to  
10 express the avian gene.

11 V. Introduction of Gene Sequences into Somatic Cells  
12

13 DNA has been introduced into somatic cells to produc  
14 variant cell lines. hprt-deficient Chinese hamster ovary  
15 (CHO) cells have been transformed with the CHO hprt gene in  
16 order to produce a prototrophic cell line (Graf, L.H. et  
17 al., Somat. Cell Genet. 5:1031-1044 (1979)). Folger et al.  
18 examined the fate of a thymidine kinase gene (tk gene) which  
19 had been microinjected into the nuclei of cultured mammalian  
20 cells. Recipient cells were found to contain from 1 to 100  
21 copies of the introduced gene sequence integrated as  
22 concatemers at one or a few sites in the cellular genome  
23 (Folger, K.R. et al., Molec. Cell. Biol. 2:1372-1387  
24 (1982)). DNA-mediated transformation of an RNA polymeras  
25 II gene into Syrian hamster cells has also been reported  
26 (Ingles, C. et al., Molec. Cell. Biol. 2:666-673 (1982)).

27 Plasmids conferring host neomycin resistance and  
28 guanosine phosphotransferase activity have been transfected  
29 into Chinese hamster ovary cells to generate novel cell  
30 lines (Robson, C.N. et al., Mutat. Res. 163:201-208 (1986)).  
31  
32

## VI. Chimeric r Transgenic Plants

Extensive progress has been made in recent years in the fields of plant cell genetics and gene technology. For many genera of plants, protoplast regeneration techniques can be used to regenerate a plant from a single cell (Friedt, W. et al., Prog. Botany 49:192-215 (1987); Brunold, C. et al., Molec. Gen. Genet. 208:469-473 (1987); Durand, J. et al., Plant Sci. 62:263-272 (1989) which references are incorporated herein by reference).

Several methods can be used to deliver and express a foreign gene into a plant cell. The most widely used method employs cloning the desired gene sequence into the Ti plasmid of the soil bacterium A. tumorigens (Komari, T. et al., J. Bacteriol. 166:88-94 (1986); Czako, M. et al., Plant Mol. Biol. 6:101-109 (1986); Jones, J.D.G. et al., EMBO J. 4:2411-2418 (1985); Shahin, E.A. et al., Theor. Appl. Genet. 73:164-169 (1986)). The frequency of transformation may be as high as 70%, depending upon the type of plant used (Friedt, W. et al., Prog. Botany 49:192-215 (1987)).

Plant viruses have also been exploited as vectors for the delivery and expression of foreign genes in plants. The cauliflower mosaic virus (Brisson, N. et al., Nature 310:511-514 (1984) has been particularly useful for this purpose (Shah, D.M. et al., Science 233:478-481 (1986); Shewmaker, C.K. et al., Viol. 140:281-288 (1985). Vectors have also been prepared from derivatives of RNA viruses (French, R. et al., Science 231:1294-1297 (1986)).

Techniques of microinjection (Crossway, A. et al., Molec. Gen. Genet. 202:179-185 (1986); Potrykus, I. et al., Molec. Gen. Genet. 199:169-177 (1985)), have been used to accomplish the direct transfer of gene sequences into plant

1 c lls. Transformation with a plasmid capable of site  
2 specific recombination has been used to introduce gene  
3 sequences into Aspergillus (May, G.S., J. Cell Biol.  
4 109:2267-2274 (1989); which reference is incorporated herein  
5 by reference).

6 Electroporation has been identified as a method for  
7 introducing DNA into plant cells (Fromm, M.E., et al., Proc.  
8 Natl. Acad. Sci. (U.S.A.) 82:5824-5828 (1985); Fromm, M.E.  
9 et al., Nature 319:791-793 (1986); Morikawa, H. et al., Gene  
10 41:121-124 (1986); Langridge, W.H.R. et al., Theor. Appl.  
11 Genet. 67:443-455 (1984)).

12 Gross genetic mutations can be produced in plant cells  
13 using transposable elements (Saedler, H. et al., EMBO J.  
14 4:585-590 (1985); Peterson, P.A., BioEssays 3:199-204  
15 (1985)). Such elements can initiate chromosomal  
16 rearrangements, insertions, duplications, deletions, etc.  
17 Chimeric plants can be regenerated from such cells using the  
18 procedures described above.

19 A major deficiency of present methods for gen  
20 manipulation in plants is the difficulty of selecting the  
21 desired recombinant cell (Brunold, C. et al., Molec. Gen.  
22 Genet. 208:469-473 (1987)). In an attempt to address this  
23 deficiency, kanamycin resistance and nitrate reductase  
24 deficiency have been used as selectable markers (Brunold, C.  
25 et al., Molec. Gen. Genet. 208:469-473 (1987)).  
26  
27

## VII. C nclusi ns

The applicati n f the abov -describ d technologies has the potential to produce types of plants and animals which cannot be produced through classical genetics. For example, animals can be produced which suffer from human diseases (such as AIDS, diabetes, cancer, etc.), and may be valuable in elucidating therapies for such diseases. Chimeric and transgenic plants and animals have substantial use as probes of natural gene expression. When applied to livestock and food crops, the technologies have the potential of yielding improved food, fiber, etc.

Despite the successes of the above-described techniques, a method for producing chimeric or transgenic plants and animals which was less mutagenic, and which would permit defined, specific, and delicate manipulation of the inserted gene sequence at a specific chromosomal location would be highly desirable.

### BRIEF DESCRIPTION OF THE FIGURES:

Figure 1 illustrates the use of replacement vectors and insertion vectors in gene targeting. Figure 1A is a diagrammatical representation of the use of a replacement vector in gene targeting; Figure 1B illustrates the use f an insertion vector to produce subtle mutations in a desired gene sequence.

Figure 2 is a diagrammatical representation of a DNA molecule which has a region of heterology located at a proposed insertion site. Figure 2A shows a construct with a 2 kb region of heterology. Figure 2B shows a construct with a 26 base long region of heterology which has been

linearized at the center of the region of heterology. Figure 2C shows a construct with a region of heterology located internal to the region of homology at which recombination is desired. In Figure 2C, the normal BamHI site of the vector has been changed to an NheI site and the normal EcoRI site of the vector has been changed to a BamHI site. The vector is linearized with XhoI.

Figure 3 is a diagrammatical representation of the mechanism through which a "humanized" gene may be introduced into a chromosomal gene sequence in a one step method.

Figure 4 is a diagrammatical representation of the mechanism through which a large gene may be introduced into a chromosomal gene sequence so as to place the gene under the transcriptional control of a heterologous promoter (for example, to place a human gene under the control of a mouse gene). The first step is additive and the second is a replacement event. Figure 4A shows the first step of the process; Figure 4B shows the second step of the process. The repair recombination event may be configured to remove all of the mouse coding exons if desired.

Figure 5 is a diagrammatical representation of the use of a positive selection/ negative selection "cassette" to introduce subtle mutations into a chromosome.

Figure 6 is a diagrammatical representation of a multi-step method (Figures 6A-6E) for introducing small or large desired gene sequences into a contiguous region of a cell's genome. The figure illustrates a vector capable of facilitating the sequential addition of overlapping clones to construct a large locus. Every step is selectable. Subsequent additions may be made by returning to steps 4 and 5 as many times as required, selecting for insertion in HAT medium, and for repair in media supplemented with 6

1 thioguanine. This procedure may also be accomplished at the  
2 ther end of the locus if required.

3 Figure 7 is a diagrammatical representation of the  
4 vectors used in a co-electroporation experiment to mutate  
5 the hprt gene.

6 Figure 8 illustrates the predicted structure of the hprt  
7 gene following homologous recombination of the IV6.8 vector.  
8 HR is the predicted size fragment indicative of the  
9 homologous recombination event. End, D is the endogenous  
10 fragment, duplicated by the recombination event. End is the  
11 predicted flanking fragment detected by the partial cDNA  
12 probe used in these experiments.

13 Figure 9 shows the reversion of homologous recombinants  
14 generated with insertion vectors.

15 Figure 10 illustrates the use of Poly A selection as a  
16 means for selecting homologous recombination events.

17 Figure 11 illustrates the use of the invention to  
18 introduce insertions into the sequence of a desired gene of  
19 a cell. Figure 11A is a diagram of the c-src locus showing  
20 relevant restriction sites (E=EcoRI; N=NcoI; X=XhoI;  
21 H=HindIII; B=BamHI; Nh=NheI). Figure 11B illustrates the  
22 src 14 vector used to introduce mutations into the c-src  
23 locus; Figure 11C illustrates the subtle mutation introduced  
24 through the use of this vector.

25 Figure 12 illustrates the use of the invention to  
26 introduce substitutions into the sequence of a desired gene  
27 of a cell. Figure 12A is a diagram of the c-src locus  
28 showing relevant restriction sites (E=EcoRI; N=NcoI; X=XhoI;  
29 H=HindIII; B=BamHI; Nh=NheI). Figure 12B illustrates the  
30 src 33 vector used to introduce mutations into the c-src  
31 locus; Figure 12C illustrates the subtle mutation introduced  
32 through the use of this vector.



1           Figure 13 illustrates a comparison between targeted and  
2 random recombinational events. In a random recombinational  
3 event, although concatemers can excise duplications, on  
4 copy of the vector must remain in the genome. In contrast,  
5 in a targeted recombinational event, all sequences, except  
6 the desired sequence is excised from the genome.

7  
8           SUMMARY OF THE INVENTION:

9  
10           The present invention provides a method for obtaining a  
11 desired animal or non-fungal plant cell which contains a  
12 predefined, specific and desired alteration in its genome.  
13 The invention further pertains to the non-human animals and  
14 plants which may be produced from such cells. The invention  
15 additionally pertains to the use of such non-human animals  
16 and plants, and their progeny in research, medicine, and  
17 agriculture.

18           In detail, the invention provides a method for obtaining  
19 a desired animal or non-fungal plant cell which contains a  
20 desired non-selectable gene sequence inserted within a  
21 predetermined gene sequence of the cell's genome, which  
22 method comprises:

23           A. incubating a precursor cell with a DNA molecule  
24 containing the desired non-selectable gene sequence, where in  
25 the DNA molecule additionally contains two regions of  
26 homology which flank the desired gene sequence, and which  
27 are sufficient to permit the desired gene sequence to  
28 undergo homologous recombination with the predetermined gene  
29 sequence of the genome of the precursor cell;

30           B. causing the DNA molecule to be introduced into  
31 the precursor cell;

32           C. permitting the introduced DNA molecule to  
33 undergo homologous recombination with the predetermined gene

1 sequence of the genome of the precursor cell to thereby  
2 produce the desired cell wherein the desired non-selectable  
3 gene sequence has been inserted into the predetermined gene  
4 sequence; and

5 D. recovering the desired cell.

6 The invention further includes the embodiments of the  
7 above-described method wherein the DNA molecule contains a  
8 detectable marker gene sequence, and/or wherein the DNA  
9 molecule is introduced into the precursor cell by subjecting  
10 the precursor cell and the DNA molecule to electroporation  
11 (especially wherein in step B, the precursor cell is  
12 simultaneously subjected to electroporation with a second  
13 DNA molecule, the second DNA molecule containing a  
14 detectable marker gene sequence).

15 The invention further includes the embodiments of the  
16 above-described method wherein the desired cell is a non-  
17 fungal plant cell, a somatic animal cell (especially one  
18 selected from the group consisting of a chicken, a mouse, a  
19 rat, a hamster, a rabbit, a sheep, a goat, a fish, a pig, a  
20 cow or bull, a non-human primate and a human), a pluripotent  
21 animal cell (especially one selected from the group  
22 consisting of a chicken, a mouse, a rat, a hamster, a  
23 rabbit, a sheep, a goat, a fish, a pig, a cow or bull, and  
24 a non-human primate). The invention includes with the  
25 embodiment wherein the pluripotent cell is an embryonic stem  
26 cell.

27 The invention also includes the embodiments of the  
28 above-described methods wherein the desired gene sequence is  
29 substantially homologous to the predetermined gene sequence  
30 of the precursor cell and/or wherein the desired gene  
31 sequence is an analog (and especially a human analog) of the  
32 predetermined sequence of the precursor cell.

1           The invention also includes the embodiment wherein the  
2           desired gene sequence encodes a protein selected from the  
3           group consisting of: a hormone, an immunoglobulin, a  
4           receptor molecule, a ligand of a receptor molecule, and an  
5           enzyme.

6           The invention also includes a non-fungal plant cell  
7           which contains an introduced recombinant DNA molecule  
8           containing a desired gene sequence, the desired gene  
9           sequence being flanked by regions of homology which are  
10          sufficient to permit the desired gene sequence to undergo  
11          homologous recombination with a predetermined gene sequence  
12          of the genome of the cell.

13          The invention also includes a non-human animal cell  
14          which contains an introduced recombinant DNA molecule  
15          containing a desired gene sequence, the desired gene  
16          sequence being flanked by regions of homology which are  
17          sufficient to permit the desired gene sequence to undergo  
18          homologous recombination with a predetermined gene sequence  
19          of the genome of the cell.

20          The invention also includes the desired cell produced by  
21          any of the above-described methods.

22          The invention also includes a non-human animal  
23          containing a cell derived from the above-described desired  
24          cell, or a descendant thereof, wherein the animal is either  
25          a chimeric or a transgenic animal, and particularly includes  
26          the embodiment wherein the non-human animal and the desired  
27          cell are of the same species, and wherein the species is  
28          selected from the group consisting of: a chicken, a mouse,  
29          a rat, a hamster, a rabbit, a sheep, a goat, a fish, a pig,  
30          a cow or bull, and a non-human primate.

31          The invention also includes a non-fungal plant  
32          containing a cell derived from the above-described desired

1       n n-fungal plant cell, wher in said n n-fungal plant is  
2       either a chimeric r a transgenic plant.

3       The invention also includes a method of gen th rapy  
4       which comprises introducing to a recipient in need of such  
5       therapy, a desired non-selectable gene sequence, the method  
6       comprising:

7           A. providing to the recipient an effective amount  
8       of a DNA molecule containing the desired non-selectable gene  
9       sequence, wherein the DNA molecule additionally contains two  
10      regions of homology which flank the desired gene sequence,  
11      and which are sufficient to permit the desired gene sequence  
12      to undergo homologous recombination with a predetermin d  
13      gene sequence present in a precursor cell of the recipient;

14           B. permitting the DNA molecule to be introduced  
15      into the precursor cell;

16           C. permitting the introduced DNA molecule t  
17      undergo homologous recombination with the predetermined gen  
18      sequence of the genome of the precursor cell to thereby  
19      produce a desired cell wherein the desired non-selectable  
20      gene sequence has been inserted into the predetermined g n  
21      sequence; and wherein the presence or expression of th  
22      introduced gene sequence in the cell of the recipient  
23      comprises the gene therapy.

24       In particular, the invention includes the embodiments of  
25      the above-stated method wherein the recipient is a non-  
26      fungal plant, or a human or a non-human animal (particularly  
27      a non-human animal is selected from the group consisting of:  
28      a chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a  
29      goat, a fish, a pig, a cow or bull, a non-human primate and  
30      a human).

1       The invention also provides a method for obtaining a  
2 desired animal or non-fungal plant cell which contains a  
3 desired non-selectable gene sequence inserted within a  
4 predetermined gene sequence of the cell's genome, which  
5 method comprises:

6       A. incubating a precursor cell under non-selective  
7 culture conditions, or under a first set of selective  
8 culture conditions, with a DNA molecule containing:

9       i) the desired non-selectable gene sequence,  
10 wherein the DNA molecule additionally contains  
11 two regions of homology which flank the desired  
12 gene sequence, and which are sufficient to  
13 permit the desired gene sequence to undergo  
14 homologous recombination with the predetermined  
15 gene sequence of the genome of the precursor  
16 cell; and

17       ii) a selectable gene sequence whose presence or  
18 expression in the cell can be selected for by  
19 culturing the cells under the first set of  
20 selective culture conditions, and whose  
21 presence or expression in the cell can be  
22 selected against by culturing the cells under  
23 a second set of selective culture conditions;

24       B. permitting the DNA molecule to be introduced  
25 into the precursor cell;

26       C. permitting the introduced DNA molecule to  
27 undergo homologous recombination with the predetermined gene  
28 sequence of the genome of the precursor cell to thereby  
29 produce the desired cell wherein the desired non-selectable  
30 gene sequence has been inserted into the predetermined gene  
31 sequence; and

32       D. recovering the desired cell by culturing the  
33 cell under the first set of selective culture conditions, by

1 then permitting the cell to undergo intrachromosomal  
2 recombination under non-selective culture conditions, and by  
3 then incubating the cell under the second set of selective  
4 culture conditions.

5 The invention also includes the embodiment wherein the  
6 cell is deficient in an HPRT, APRT, or TK enzyme, and  
7 wherein the selectable gene sequence expresses an active  
8 HPRT, APRT, or TK enzyme, and wherein the first set of  
9 selective culture conditions comprises incubation of the  
10 cell under conditions in which the presence of an active  
11 HPRT, APRT, or TK enzyme in the cell is required for growth,  
12 and wherein the second set of selective culture conditions  
13 comprises incubation of the cell under conditions in which  
14 the absence of an active HPRT, APRT, or TK enzyme in the  
15 cell is required for growth.

16  
17  
18 DESCRIPTION OF THE PREFERRED EMBODIMENTS:

19  
20 The present invention concerns a method for introducing  
21 DNA into the genome of a recipient plant or animal cell.  
22 The method may be used to introduce such DNA into germ line  
23 cells of animals (especially, rodents (i.e. mouse, rat,  
24 hamster, etc.), rabbits, sheep, goats, fish, pigs, cattle  
25 and non-human primates) in order to produce chimeric or  
26 transgenic animals. The methods may also be used to  
27 introduce DNA into plant cells which can then be manipulated  
28 in order to produce chimeric or transgenic plants.

29 Alternatively, the method may be used to alter the  
30 somatic cells of an animal (including humans) or a plant.  
31 The plants and plant cells which may be manipulated through  
32 application of the disclosed method include all  
33 multicellular, higher (i.e. non-fungal or non-yeast) plants.

# I. Homologous Recombination

The present invention provides a method for introducing a desired gene sequence into a plant or animal cell. Thus, it is capable of producing chimeric or transgenic plants and animals having defined, and specific, gene alterations.

An understanding of the process of homologous recombination (Watson, J.D., In: Molecular Biology of the Gene, 3rd Ed., W.A. Benjamin, Inc., Menlo Park, CA (1977), which reference is incorporated herein by reference) is desirable in order to fully appreciate the present invention.

In brief, homologous recombination is a well-studied natural cellular process which results in the scission of two nucleic acid molecules having identical or substantially similar sequences (i.e. "homologous"), and the ligation of the two molecules such that one region of each initially present molecule is now ligated to a region of the other initially present molecule (Sedivy, J.M., Bio-Technol. 6:1192-1196 (1988), which reference is incorporated herein by reference).

Homologous recombination is, thus, a sequence specific process by which cells can transfer a "region" of DNA from one DNA molecule to another. As used herein, a "region" of DNA is intended to generally refer to any nucleic acid molecule. The region may be of any length from a single base to a substantial fragment of a chromosome.

For homologous recombination to occur between two DNA molecules, the molecules must possess a "region of homology" with respect to one another. Such a region of homology must be at least two base pairs long. Two DNA molecules possess such a "region of homology" when one contains a region whos

1        sequen    is s   similar to a region in the second molecule  
2        that hom log us rec mbinati n can occur.

3        Recombinati n    is catalyzed by enzym s    which are  
4        naturally present in both prokaryotic and eukaryotic cells.  
5        The transfer of a region of DNA may be envisioned as  
6        occurring through a multi-step process.

7        If either of the two participant molecules is a circular  
8        molecule, then the above recombination event results in the  
9        integration of the circular molecule into the other  
10       participant.

11       Importantly, if a particular region is flanked by  
12       regions of homology (which may be the same, but ar  
13       preferably different), then two recombinational events may  
14       occur, and result in the exchange of a region of DNA between  
15       two DNA molecules. Recombination may be "reciprocal," and  
16       thus results in an exchange of DNA regions between tw  
17       recombining DNA molecules. Alternatively, it may be "non-  
18       reciprocal," (also referred to as "gene conversion") and  
19       result in both recombining nucleic acid molecules having the  
20       same nucleotide sequence.       There are no constraints  
21       regarding the size or sequence of the region which is  
22       exchanged in a two-event recombinational exchange.

23       The frequency of recombination between two DNA molecules  
24       may be enhanced by treating the introduced DNA with agents  
25       which stimulate recombination.       Examples of such agents  
26       include trimethylpsoralen, UV light, etc.

27



1                   II.           Pr ducti n f Chimeric and Transgenic Animals:  
2                   Gen Targeting Methods

3  
4           One approach to producing animals having defined and  
5           specific genetic alterations has used homologous  
6           recombination to control the site of integration of an  
7           introduced marker gene sequence in tumor cells and in  
8           fusions between diploid human fibroblast and tetraploid  
9           mouse erythroleukemia cells (Smithies, O. et al., Nature  
10           317:230-234 (1985)).

11           This approach was further exploited by Thomas, K. R.,  
12           and co-workers, who described a general method, known as  
13           "gene targeting," for targeting mutations to a preselected,  
14           desired gene sequence of an ES cell in order to produce a  
15           transgenic animal (Mansour, S.L. et al., Nature 336:348-352  
16           (1988); Capecchi, M.R. Trends Genet. 5:70-76 (1989);  
17           Capecchi, M.R. et al., In: Current Communications in  
18           Molecular Biology, Capecchi, M.R. (ed.), Cold Spring Harbor  
19           Press, Cold Spring Harbor, NY (1989), pp. 45-52; which  
20           references are incorporated herein by reference).

21           Gene targeting has been used to produce chimeric and  
22           transgenic mice in which an nptII gene has been inserted  
23           into the  $\beta_2$ -microglobulin locus (Koller, B.H. et al., Proc.  
24           Natl. Acad. Sci. (U.S.A.) 86:8932-8935 (1989); Zijlstra, M.  
25           et al., Nature 342:435-438 (1989); Zijlstra, M. et al.,  
26           Nature 344:742-746 (1989); DeChiaba et al., Nature 345:78-80  
27           (1990)). Similar experiments have enabled the production of  
28           chimeric and transgenic animals having a c-abl gene which  
29           has been disrupted by the insertion of an nptII gene  
30           (Schwartzberg, P.L. et al., Science 246:799-803 (1989)).  
31           The technique has been used to produce chimeric mice in  
32           which the en-2 gene has been disrupted by the insertion of

1 an nptII gen (Joyner, A.L. et al., Nature 338:153-155  
2 (1989)).

3 Gen targ ting has also be n us d to corr ct an hprt  
4 deficiency in an hprt ES cell line. Cells corrected of the  
5 deficiency were used to produce chimeric animals.  
6 Significantly, all of the corrected cells exhibited gross  
7 disruption of the regions flanking the hprt locus; all f  
8 the cells tested were found to contain at least one copy f  
9 the vector used to correct the deficiency, integrated at the  
10 hprt locus (Thompson, S. et al., Cell 56:313-321 (1989);  
11 Koller, B.H. et al., Proc. Natl. Acad. Sci. (U.S.A.)  
12 86:8927-8931 (1989)).

13 In order to utilize the "gene targeting" method, the  
14 gene of interest must have been previously cloned, and the  
15 intron-exon boundaries determined. The method results in  
16 the insertion of a marker gene (i.e. the nptII gene) into a  
17 translated region of a particular gene of interest. Thus,  
18 use of the gene targeting method results in the gross  
19 destruction of the gene of interest.

20 Recently, chimeric mice carrying the homeobox hox 1.1  
21 allele have been produced using a modification of the gene  
22 targeting method (Zimmer, A. et al., Nature 338:150-154  
23 (1989). In this modification, the integration of vect r  
24 sequences was avoided by microinjecting ES cells with linear  
25 DNA containing only a portion of the hox 1.1 allele, without  
26 any accompanying vector sequences. The DNA was found t  
27 cause the gene conversion of the cellular hox allele.  
28 Selection was not used to facilitate the recovery of the  
29 "converted" ES cells, which were identified using the  
30 polymerase chain reaction ("PCR"). Approximately 50% of  
31 cells which had been clonally purified from "converted"  
32 cells were found to contain the introduced hox 1.1 allel ,  
33 suggesting to Zimmer, A. et al. either chromosomal

1 instability or contamination of sample. None of the  
2 chimeric microwere found to be able to transmit the  
3 "converted" gene to their progeny (Zimmer, A. et al., In:  
4 Current Communications in Molecular Biology, Capecchi, M.R.  
5 (ed.), Cold Spring Harbor Press, Cold Spring Harbor, NY  
6 (1989), pp. 53-58).

7 The use of the gene targeting method is illustrated in  
8 Figure 1A. In that figure, a gene construct is produced in  
9 which the nptII gene is inserted into an exon (designated  
10 region "3") of a sequence of the hprt gene. The construct  
11 is then permitted to undergo recombination with the hprt  
12 gene of a cell. Such recombination results in the  
13 replacement of the exon 3 sequence of the cell with the  
14 disrupted exon 3 - nptII sequence of the construct.  
15 Significantly, as illustrated in Figure 1A, the use of gene  
16 targeting to alter a gene of a cell results in the formation  
17 of a gross alteration in the sequence of that gene. As  
18 indicated in Figure 1A, the efficiency of gene targeting is  
19 approximately 1/300.

### 20 III. Production of Chimeric and Transgenic Animals: 21 Use of Insertion Vectors 22

23 In contrast to the above-described methods, the present  
24 invention is capable of producing subtle, precise, and  
25 predetermined mutations in the sequence of a desired gene of  
26 a cell. The present invention has several embodiments, the  
27 simplest of which is illustrated in Figure 1B.

28 As shown in Figure 1B, an insertion vector is used to  
29 mutate the nucleotide sequence of the hprt gene. The use of  
30 this vector type in combination with a second selectable  
31 reversion event prevents the disruption of the chromosome by  
32 the nptII gene or by the vector sequences. Thus, gross  
33

1       dist rti ns of th r cipi nt chr m som are av ided by the  
2       present inventi n. Mor over, the efficiency of the gene  
3       targeting was substantially improv d (i.e. 1/32 as opposed  
4       to 1/300).

5       The DNA molecule(s) which are to be introduced into th  
6       recipient cell preferably contains a region of homology with  
7       a region of the cellular genome. In a preferred embodiment,  
8       the DNA molecule will contain two regions of homology with  
9       the genome (both chromosomal and episomal) of the  
10      pluripotent cell. These regions of homology will preferably  
11      flank a "desired gene sequence" whose incorporation into the  
12      cellular genome is desired. As stated above, the regions of  
13      homology may be of any size greater than two bases long.  
14      Most preferably, the regions of homology will be greater  
15      than 10 bases long.

16      The DNA molecule(s) may be single stranded, but are  
17      preferably double stranded. The DNA molecule(s) may be  
18      introduced to the cell as one or more RNA molecules which  
19      may be converted to DNA by reverse transcriptase or by other  
20      means. Preferably, the DNA molecule will be double stranded  
21      linear molecule. In the best mode for conducting the  
22      invention, such a molecule is obtained by cleaving a clos d  
23      covalent circular molecule to form a linear molecule.  
24      Preferably, a restriction endonuclease capable of cleaving  
25      the molecule at a single site to produce either a blunt end  
26      or staggered end linear molecule is employed. Most  
27      preferably, the nucleotides on each side of this restriction  
28      site will comprise at least a portion of the preferred two  
29      regions of homology between the DNA molecule being  
30      introduced and the cellular genome.

31      The invention thus provides a method for introducing the  
32      "desired gene sequence" into the genome of an animal r  
33      plant at a specific chromosomal location. The "desired gen

1        sequenc " may be f any length, and have any nucleotide  
2        s qu nc . It may c mprise ne r more g n sequences which  
3        encode complete pr t ins, fragments f such gen sequences,  
4        regulatory sequences, etc. Significantly, the desired gene  
5        sequence may differ only slightly from a native gene of the  
6        recipient cell (for example, it may contain single, or  
7        multiple base alterations, insertions or deletions relative  
8        to the native gene). The use of such desired gene sequences  
9        will permit one to create subtle and precise changes in the  
10       genome of the recipient cell. Thus, the present invention  
11       provides a means for manipulating and modulating gen  
12       expression and regulation.

13       In particular, the invention provides a mean for  
14       manipulating and modulating gene expression and protein  
15       structure through the replacement of a gene sequence with a  
16       "non-selectable" "desired gene sequence." A gene sequenc  
17       is non-selectable if its presence or expression in a  
18       recipient cell provides no survival advantage to the cell  
19       under the culturing conditions employed. Thus, by  
20       definition, one cannot select for cells which have receiv d  
21       a "non-selectable" gene sequence. In contrast, a "dominant"  
22       gene sequence is one which can under certain circumstanc s  
23       provide a survival advantage to a recipient cell. The  
24       neomycin resistance conferred by the nptII gene is a  
25       survival advantage to a cell cultured in the presence of  
26       neomycin or G418. The nptII gene is thus a dominant, rather  
27       than a non-selectable gene sequence.

28       In particular, the invention permits the replacement of  
29       a gene sequence which is present in the recipient cell with  
30       an "analog" sequence. A sequence is said to be an analog of  
31       another sequence if the two sequences are substantially  
32       similar in sequence, but have minor changes in sequ nc  
33       corresponding to single base substitutions, deletions, or

1 insertions with respect to on another, or if they possess  
2 "minor" multiple base alterations. Such alterations are  
3 intended to exclude insertions of dominant selectable marker  
4 genes.

5 When the desired gene sequence, flanked by regions of  
6 homology with the recipient cell, is introduced into the  
7 recipient cell as a linear double stranded molecule, whose  
8 termini correspond to the regions of homology, a single  
9 recombination event with the cell's genome will occur in  
10 approximately 5% of the transfected cells. Such a single  
11 recombinational event will lead to the integration of the  
12 entire linear molecule into the genome of the recipient  
13 cell.

14 The structure generated by the integration of the linear  
15 molecule will undergo a subsequent, second recombinational  
16 event (approximately  $10^{-5}$  -  $10^{-7}$  per cell generation). This  
17 second recombinational event will result in the elimination  
18 of all DNA except for the flanking regions of homology, and  
19 the desired DNA sequence from the integrated structure.

20 Thus, the consequence of the second recombinational event  
21 is to replace the DNA sequence which is normally present  
22 between the flanking regions of homology in the cell's  
23 genome, with the desired DNA sequence, and to eliminate the  
24 instability of gene replacement.

25 The DNA molecule containing the desired gene sequence  
26 may be introduced into the pluripotent cell by any method  
27 which will permit the introduced molecule to undergo  
28 recombination at its regions of homology. Some methods,  
29 such as direct microinjection, or calcium phosphate  
30 transformation, may cause the introduced molecule to form  
31 concatemers upon integration. These concatemers may resolve  
32 themselves to form non-concatemeric integration structures.  
33 Since the presence of concatemers is not desired, methods

1 which produce them are not preferred. In a preferred  
2 embodiment, the DNA is introduced by electroporation  
3 (Toneguzzo, F. et al., Nucleic Acids Res. 16:5515-5532  
4 (1988); Quillet, A. et al., J. Immunol. 141:17-20 (1988);  
5 Machy, P. et al., Proc. Natl. Acad. Sci. (U.S.A.) 85:8027-  
6 8031 (1988); all of which references are incorporated herein  
7 by reference).

8 After permitting the introduction of the DNA  
9 molecule(s), the cells are cultured under conventional  
10 conditions, as are known in the art.

11 In order to facilitate the recovery of those cells which  
12 have received the DNA molecule containing the desired gene  
13 sequence, it is preferable to introduce the DNA containing  
14 the desired gene sequence in combination with a second gene  
15 sequence which would contain a detectable marker gene  
16 sequence. For the purposes of the present invention, any  
17 gene sequence whose presence in a cell permits one to  
18 recognize and clonally isolate the cell may be employed as  
19 a detectable marker gene sequence.

20 In one embodiment, the presence of the detectable marker  
21 sequence in a recipient cell is recognized by hybridization,  
22 by detection of radiolabelled nucleotides, or by other  
23 assays of detection which do not require the expression of  
24 the detectable marker sequence. Preferably, such sequences  
25 are detected using PCR (Mullis, K. et al., Cold Spring  
26 Harbor Symp. Quant. Biol. 51:263-273 (1986); Erlich H. et  
27 al., EP 50,424; EP 84,796, EP 258,017, EP 237,362; Mullis,  
28 K., EP 201,184; Mullis K. et al., US 4,683,202; Erlich, H.,  
29 US 4,582,788; and Saiki, R. et al., US 4,683,194), which  
30 references are incorporated herein by reference).

31 PCR achieves the amplification of a specific nucleic  
32 acid sequence using two oligonucleotide primers  
33 complementary to regions of the sequence to be amplified.

1 Extension products incorporating the primers then become  
2 templates for subsequent replication steps. PCR provides a  
3 method for selectively increasing the concentration of a  
4 nucleic acid molecule having a particular sequence even when  
5 that molecule has not been previously purified and is  
6 present only in a single copy in a particular sample. The  
7 method can be used to amplify either single or double  
8 stranded DNA.

9 Most preferably, however, the detectable marker gene  
10 sequence will be expressed in the recipient cell, and will  
11 result in a selectable phenotype. Examples of such  
12 preferred detectable gene sequences include the hprt gene  
13 (Littlefield, J.W., Science 145:709-710 (1964), herein  
14 incorporated by reference), a xanthine-guanine  
15 phosphoribosyltransferase (gpt) gene, or an adenosine  
16 phosphoribosyltransferase (aprt) gene (Sambrook et al., In:  
17 Molecular Cloning A Laboratory Manual, 2nd. Ed., Cold Spring  
18 Harbor Laboratory Press, NY (1989), herein incorporated by  
19 reference), a tk gene (i.e. thymidine kinase gene) and  
20 especially the tk gene of herpes simplex virus (Giphart-  
21 Gassler, M. et al., Mutat. Res. 214:223-232 (1989) herein  
22 incorporated by reference), the npvII gene (Thomas, K.R. et  
23 al., Cell 51:503-512 (1987); Mansour, S.L. et al., Nature  
24 336:348-352 (1988), both references herein incorporated by  
25 reference), or other genes which confer resistance to amino  
26 acid or nucleoside analogues, or antibiotics, etc. Examples  
27 of such genes include gene sequences which encode enzymes  
28 such as dihydrofolate reductase (DHFR) enzyme, adenosine  
29 deaminase (ADA), asparagine synthetase (AS), hygromycin B  
30 phosphotransferase, or a CAD enzyme (carbamyl phosphate  
31 synthetase, aspartate transcarbamylase, and dihydroorotase)  
32 (Sambrook et al., In: Molecular Cloning A Laboratory Manual,



2nd. Ed., Cold Spring Harbor Laboratory Press, NY (1989),  
herein incorporated by reference).

Cells that do not contain an active thymidine kinase  
(TK) enzyme, a hypoxanthine-phosphoribosyltransferase (HPRT)  
enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)  
enzyme, or an adenosine phosphoribosyltransferase (APRT)  
enzyme, are unable to grow in medium containing  
hypoxanthine, aminopterin, and/or mycophenolic acid (and  
preferably adenine, xanthine, and/or thymidine), and  
thymidine, but are able to grow in medium containing  
nucleoside analogs such as 5-bromodeoxyuridine, 6-  
thioguanine, 8-azapurine, etc. (Littlefield, J.W., Science  
145:709-710 (1964); Sambrook et al., In: Molecular Cloning  
A Laboratory Manual, 2nd. Ed., Cold Spring Harbor Laboratory  
Press, NY (1989)).

Conversely, cells that do contain such active enzymes  
are able to grow in such medium, but are unable to grow in  
medium containing nucleoside analogs such as 5-  
bromodeoxyuridine, 6-thioguanine, 8-azapurine, etc.  
(Sambrook et al., In: Molecular Cloning A Laboratory Manual,  
2nd. Ed., Cold Spring Harbor Laboratory Press, NY (1989)).

Cells expressing active thymidine kinase are able to  
grow in media containing HATG, but are unable to grow in  
media containing nucleoside analogues such as 5-azacytidine  
(Giphart-Gassler, M. et al., Mutat. Res. 214:223-232  
(1989)). Cells containing an active HSV-tk gene are  
incapable of growing in the presence of gangcylovir or  
similar agents.

The detectable marker gene may be any gene which can  
complement for a recognizable cellular deficiency. Thus,  
for example, the gene for HPRT could be used as the  
detectable marker gene sequence when employing cells lacking  
HPRT activity. Thus, this gene is an example of a gene

1       wh se expressi n product may be used to select mutant cells,  
2       or t "n gatively sele t" for cells which express this gene  
3       product.

4       The nptII gene (Southern, P.J., et al., J. Molec. Appl.  
5       Genet. 1:327-341 (1982); Smithies, O. et al., Nature  
6       317:230-234 (1985), which references are incorporated herein  
7       by reference) is the most preferred detectable marker gene  
8       sequence. Constructs which contain both an nptII gene and  
9       either a tk gene or an hprt gene are especially preferred.

10  
11       A. Use of a Single DNA Molecule Containing Both th  
12       Detectable Marker Sequence and the Desired Gene  
13       Sequence

14  
15       In a first preferred embodiment, the detectable marker  
16       gene sequence, flanked by the regions of homology, is  
17       provided to the recipient cells on the same DNA molecule  
18       which contains the desired gene sequence. As discuss d  
19       previously, it is preferred that this DNA molecule be a  
20       linear molecule.

21       After selection for cells which have incorporated the  
22       desired DNA molecule (for example by selection for G418  
23       resistant cells when the detectable marker gene sequence is  
24       an expressible nptII gene sequence), the cells are cultured,  
25       and the presence of the introduced DNA molecule is confirmed  
26       as described above. Approximately  $10^7$  cells are cultured and  
27       screened for cells which have undergone the second  
28       recombinational event (discussed above) resulting in the  
29       replacement of a native sequence (i.e. a gene sequence  
30       which is normally and naturally present in the recipient  
31       cell) with the desired gene sequence.

32       Any of a variety of methods may be used to identify  
33       cells which have undergone the second recombinational event.

1        Direct screening of clones, use of PCR, use of hybridization  
2        probes, etc., may all be employed for this purpose. In a  
3        preferred embodiment, the DNA molecule will, in addition to  
4        the desired gene sequence, the flanking regions of homology  
5        and the detectable marker gene sequence, contain an  
6        additional gene sequence which will permit the selection or  
7        recognition of cells which have undergone the second  
8        recombinational event. This additional gene sequence will  
9        be excised from the cell's genome as a direct consequence of  
10       the second recombinational event. Thus, gene sequences  
11       which are suitable for this purpose include any gene  
12       sequence whose loss from a cell can be detected or selected  
13       for. Examples of such "negative selection" gene sequences  
14       include the hprt gene, and the tk gene (especially the tk  
15       gene of herpes simplex virus).

16       In the first preferred embodiment, the frequency of the  
17       second recombinational event is approximately  $10^{-5}$ . However,  
18       the use of a "negative selection" gene sequence permits one  
19       to identify such recombinant cells at a frequency of  
20       approximately 100%.

21       As illustrated in Figure 2, the DNA molecule may have a  
22       region of heterology located at the proposed insertion site.  
23       Insertion of such a vector permits one to select for  
24       recombinants which have recombined at the insertion site  
25       (and not at other potential sites). If recombination occurs  
26       at the desired insertion site, it will lead to the loss of  
27       the sequence of heterology located at the proposed insertion  
28       site of the DNA molecule (HSVtk, for example, in Figure 2A).  
29       Insertions which result from other recombinational events  
30       will retain the sequence of heterology. Thus, by employing  
31       a region of heterology which encodes an assayable gene  
32       product, or which can be used as a "negative selectable"  
33       marker, one can readily determine that the locus of

1 insertion of the recipient cell contains the precise  
2 sequence desired. As indicated in Figure 2A), the  
3 efficiency of such a vector is approximately 1/197.

4 The region of heterology which may be introduced at the  
5 insertion site of the DNA molecule may be either short (for  
6 example, 26 base pairs, Figure 2B) or of substantial size  
7 (for example, 2 kb, Figure 2A). The site of linearization  
8 may be 5', 3', or within the region of heterology. When the  
9 site of linearization is within the region of heterology,  
10 the efficiency of gene targeting is 1/63.

11 As shown in Figure 2C, the region of heterology may be  
12 located at a site internal to the region of homology where  
13 the desired recombination shall occur. Such a construct can  
14 be used when one desires to introduce a subtle mutation into  
15 a locus of the cellular gene at a site other than that of  
16 the site of desired recombination.

17  
18 B. Use of a Different DNA Molecules to Provide the  
19 Detectable Marker Sequence and the Desired Gene  
20 Sequence

21  
22 In a second preferred embodiment, the detectable marker  
23 gene sequence, flanked by the regions of homology, will be  
24 provided to the recipient cell on a different DNA molecule  
25 from that which contains the desired gene sequence. It is  
26 preferred that these molecules be linear molecules.

27 When provided on separate DNA molecules, the detectable  
28 marker gene sequence and the desired gene sequence will most  
29 preferably be provided to the recipient cell by c -  
30 electroporation, or by other equivalent techniques.

31 After selection of such recipients (preferably through  
32 the use of a detectable marker sequence which expresses the  
33 nptII gene and thus confers cellular resistance to the

antibiotic G418), the cells are grown up and screened to confirm the insertion event (preferably using PCR).

In the absence of any selection, only one cell in  $10^7$  would be expected to have the predicted recombinant structures. If, however, one selects for recipient cells which contain and express a detectable marker sequence (such as the nptII gene), it is possible to obtain a  $10^3$  to  $10^5$  fold enrichment for cells which have taken up both DNA molecules. Typically, such enrichment enables one to identify the desired recipient cell (in which the introduced DNA has integrated into the cell's genome) by screening only 800 - 1,500 cells. Such screening is preferably done using PCR, or other equivalent methods. Using such negative selection techniques, one may manipulate the vector copy number.

The two introduced DNA molecules will generally not have integrated into the same site in the genome of the recipient cell. Thus, in some cases, the desired gene sequence will have integrated in a manner so as to replace the native cellular gene sequence between the flanking regions of homology. The locus of integration of the detectable marker gene is unimportant for the purposes of the present invention, provided it is not genetically linked to the same locus as the desired gene sequence. If desired, however, it is possible to incorporate a gene sequence capable of negative selection along with the DNA containing the detectable marker sequence. Thus, one can ultimately select for cells which have lost the introduced selectable marker gene sequence DNA.

C. Use of Direct Selection to Identify Homologous  
Recombination Events

Although all of the above-described preferred embodiments enable the isolation of cells in which one of a cell's alleles has been mutated to contain a desired gene sequence, each embodiment requires the screening of a significant number of candidate cells in order to identify the desired recombinant cell. It is, however, possible to directly select for the desired recombinant cell by employing a variation of the above embodiments. This embodiment of the invention is illustrated in Figure 13. In the methods illustrated in Figure 13, if the sequence located below the asterisk is a neo gene, then only the mutant revertants will be selected if 6-thioguanine and G418 selection is applied to select for the excision events.

The method for direct selection of the desired cells relies upon the phenotypic difference in targeted and non-targeted cells and the use of a single gene which can be used for both positive and negative selection.

Typically, in any homologous recombination experiment performed with an insertion vector, three populations of cells will be created. The first class of cells will be those which have failed to receive the desired DNA molecule. This class will comprise virtually all of the candidate cells isolated on completion of the experiment. The second class of cells will be those cells in which the desired gene sequence has been incorporated at a random insertion site (i.e. a site other than in the gene desired to be mutated). Approximately one cell in  $10^3$ - $10^4$  total cells will be in this class. The third class of cells will be those cells in which the desired gene sequence has been incorporated by homologous recombination into a site in the desired gene.

1        Approximately one cell in  $10^5$ - $10^6$  total cells will be in this  
2        class.

3        In the above-described embodiments, the cells of the  
4        first class (non-transfected cells) can be eliminated by  
5        positive selection, thus necessitating the screening of only  
6        about 1,000 cells in order to identify the desired  
7        recombinant cell. In the present embodiment, cells of the  
8        third class (homologous recombinants) may be selected from  
9        the cells of the second class (random insertions) if a  
10       phenotypic difference exists between the cells of the two  
11       classes.

12       Since random integration sites are likely to be  
13       concatemeric with few single copy clones (depending upon the  
14       DNA concentration with which the cells were transfected),  
15       such integration events are inherently unstable. Thus, such  
16       concatemeric constructs will typically undergo intrachromo-  
17       somal recombination. Such recombination will always leave  
18       one intact copy of the vector in the genome. Thus, all  
19       random insertion events may be negatively selected from the  
20       population if a negatively selectable marker is included on  
21       the vector.

22       In contrast, cells in which the desired gene sequence  
23       has been incorporated into the desired gene by homologous  
24       recombination will revert with a relatively high frequency  
25       (approximately 1 in  $10^4$ - $10^5$  per cell division (depending upon  
26       the size of the duplicated structure) to produce a mutated  
27       desired gene that does not contain vector sequences.  
28       Therefore, even if the vector contained a negatively  
29       selectable gene sequence, such cells will survive negative  
30       selection, and can be recovered. The majority of homologous  
31       recombinant cells do not undergo reversion, and will be  
32       eliminated by the negative selection. Thus, the sum of the

1 selecti ns will result in th isolation of the desired  
2 r c mbinants.

3 Th method comprises incubating a "pr cursor c ll" (i.e.  
4 a cell which is to be changed by application of the method  
5 into the "desired" recombinant cell) under non-selective  
6 culture conditions, or under a first set of selectiv  
7 culture conditions. A culturing condition (i.e. medium,  
8 temperature, etc.) is said to be "non-selective" if it is  
9 capable of promoting the growth (or sustaining the  
10 viability) of a precursor cell, a desired cell, and an  
11 intermediate cell type (i.e. a cell obtained during th  
12 progression of a precursor cell into a desired cell). A  
13 culturing condition is said to be "selective" if it is  
14 capable of promoting the growth (or sustaining the  
15 viability) of only certain cells (i.e. those having a  
16 particular genotype and which therefore contain a particular  
17 gene product in either an active or an inactive form).

18 Preferred selective culturing conditions thus depend  
19 upon the genotype of the precursor cell. As stated above,  
20 cells that do not contain an active thymidine kinase (TK)  
21 enzyme, a hypoxanthine-phosphoribosyltransferase (HPRT)  
22 enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)  
23 enzyme, or an adenosine phosphoribosyltransferase (APRT)  
24 enzyme, are unable to grow in medium containing  
25 hypoxanthine, aminopterin, and/or mycophenolic acid (and  
26 preferably adenine, xanthine, and/or thymidine), and  
27 thymidine, but are able to grow in medium containing  
28 nucleoside analogs such as 5-bromodeoxyuridine, 6-  
29 thioguanine, 8-azapurine, etc. Conversely, cells that do  
30 contain such active enzymes are able to grow in such medium,  
31 but are unable to grow in medium containing nucleoside  
32 analogs such as 5-bromodeoxyuridine, 6-thioguanine, 8-  
33 azapurine, etc.



1       Such incubation is conducted in the presence of a DNA  
2       molecule containing a desired selectable gene sequence.  
3       Preferably, the DNA molecule additionally contains two  
4       regions of homology which flank this desired gene sequence,  
5       and which are sufficient to permit the desired gene sequence  
6       to undergo homologous recombination with a predetermined  
7       gene sequence of the genome of the precursor cell. The DNA  
8       molecule additionally contains a selectable gene sequence  
9       whose presence or expression in the cell can be selected for  
10      by culturing the cell under a first set of selective culture  
11      conditions, and whose presence or expression in the cell can  
12      be selected against by culturing the cell under a second set  
13      of selective culture conditions.

14      Examples of preferred selectable gene sequences include  
15      gene sequences which encode an active thymidine kinase (TK)  
16      enzyme, a hypoxanthine-phosphoribosyltransferase (HPRT)  
17      enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)  
18      enzyme, or an adenosine phosphoribosyltransferase (APRT)  
19      enzyme. Such gene sequences can be used for both positive  
20      and negative selection.

21      Additional gene sequences which can be used as  
22      selectable gene sequences include those which encode enzymes  
23      such as dihydrofolate reductase (DHFR) enzyme, adenosine  
24      deaminase (ADA), asparagine synthetase (AS), hygromycin B  
25      phosphotransferase, or a CAD enzyme (carbamyl phosphate  
26      synthetase, aspartate transcarbamylase, and dihydroorotase).  
27      Methods for producing cells deficient in expressing these  
28      enzymes are described by Sambrook et al. (In: Molecular  
29      Cloning A Laboratory Manual, 2nd. Ed., Cold Spring Harbor  
30      Laboratory Press, NY (1989), herein incorporated by  
31      reference). Such gene sequences can be used only for  
32      positive selection.

1           The incubation is performed under conditions sufficient  
2 to permit the DNA molecule to be introduced into the  
3 precursor cell. Such introduced DNA molecules are able to  
4 then undergo homologous recombination with the predetermined  
5 gene sequence of the genome of the precursor cell to ther by  
6 produce the desired cell wherein the desired non-selectable  
7 gene sequence has been inserted into the predetermined gen  
8 sequence.

9           Such a desired cell can be recovered by culturing the  
10 cell under the first set of selective culture conditions, by  
11 then permitting the cell to undergo intrachromosomal  
12 recombination under non-selective culture conditions, and by  
13 then incubating the cell under the second set of selective  
14 culture conditions.

15           Thus, in one preferred embodiment, the precursor cell  
16 lacks an active hypoxanthine-phosphoribosyltransferase (HPRT)  
17 enzyme, a xanthine-guanine phosphoribosyltransferase (XGPRT)  
18 enzyme, or an adenosine phosphoribosyltransferase (APRT)  
19 enzyme, and the selectable gene sequence expresses an active  
20 HPRT, XGPRT or APRT enzyme. In the first set of selectable  
21 culture conditions, medium containing hypoxanthin ,  
22 aminopterin and/or mycophenolic acid (and preferably  
23 adenine, xanthine, and/or thymidine) is employed. In the  
24 second set of selectable culturing conditions, medium  
25 containing a nucleoside analog such as 5-bromodeoxyuridine,  
26 6-thioguanine, 8-azapurine, etc., is employed.

27           In a second preferred embodiment, the precursor cell  
28 lacks an active TK enzyme, and the selectable gene sequence  
29 expresses an active TK enzyme. In the first set of  
30 selectable culture conditions, medium containing  
31 hypoxanthin , aminopterin, and thymidin is employed. In  
32 the second set of selectable culturing conditions, medium  
33 containing a thymidine analog such as FIAU (Borrelli, Proc.

1 Nat'l. Acad. Sci. (U.S.A.) 85:7572 (1988), or gangcyclovir,  
2 etc. is employed (if an HSV tk gene is used), or 5-  
3 bromodeoxyuridine, etc. (if a cellular tk gene is employed).

4 A preferred negative selectable marker is the hprt gene  
5 (cells expressing an active HPRT enzyme are unable to grow  
6 in the presence of certain nucleoside analogues such as 6-  
7 thioguanine, etc.). When using 6-thioguanine as a negative  
8 selection agent, a density of  $10^4$  cells /  $\text{cm}^2$  is preferably  
9 used since the efficiency of 6-thioguanine selection is cell  
10 density dependent. A typical experiment with  $10^7$  transfected  
11 cells would yield approximately 10 revertant cells after  
12 successive selection. The relative yield of revertant  
13 clones can be substantially increased by using "Poly A  
14 Selection" for the first round of selection. "Poly A  
15 Selection" is discussed in detail in Example 6 below.

#### 16 IV. The Production of Chimeric and Transgenic Animals

17  
18 The chimeric or transgenic animals of the present  
19 invention are prepared by introducing one or more DNA  
20 molecules into a precursor pluripotent cell, most preferably  
21 an ES cell, or equivalent (Robertson, E.J., In: Current  
22 Communications in Molecular Biology, Capecchi, M.R. (ed.),  
23 Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), pp.  
24 39-44, which reference is incorporated herein by reference).  
25 The term "precursor" is intended to denote only that the  
26 pluripotent cell is a precursor to the desired  
27 ("transfected") pluripotent cell which is prepared in  
28 accordance with the teachings of the present invention. The  
29 pluripotent (precursor or transfected) cell may be cultured  
30 in vivo, in a manner known in the art (Evans, M.J. et al.,  
31 Nature 292:154-156 (1981)) to form a chimeric or transgenic  
32 animal.  
33

Any ES cell may be used in accordance with the present invention. It is, however, preferred to use primary isolates of ES cells. Such isolates may be obtained directly from embryos such as the CCE cell line disclosed by Robertson, E.J., In: Current Communications in Molecular Biology, Capecchi, M.R. (ed.), Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), pp. 39-44), or from the clonal isolation of ES cells from the CCE cell line (Schwartzberg, P.A. et al., Science 246:799-803 (1989), which reference is incorporated herein by reference). Such clonal isolation may be accomplished according to the method of E.J. Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E.J. Robertson, Ed.), IRL Press, Oxford, 1987) which reference and method are incorporated herein by reference. The purpose of such clonal propagation is to obtain ES cells which have a greater efficiency for differentiating into an animal. Clonally selected ES cells are approximately 10-fold more effective in producing transgenic animals than the progenitor cell line CCE. For the purposes of the recombination methods of the present invention, clonal selection provides no advantage. An example of ES cell lines which have been clonally derived from embryos are the ES cell lines, AB1 (hprt<sup>+</sup>) or AB2.1 (hprt<sup>+</sup>).

The ES cells are preferably cultured on stomal cells (such as STO cells (especially SNC4 STO cells) and/or primary embryonic fibroblast cells) as described by E.J. Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E.J. Robertson, Ed.), IRL Press, Oxford, 1987, pp 71-112), which reference is incorporated herein by reference. The stomal (and/or fibroblast) cells serve to eliminate the clonal overgrowth of abnormal ES cells. Most preferably, the cells are cultured in th

presence of leukocyte inhibitory factor ("lif") (Gough, N.M. et al., Reprod. Fertil. Dev. 1:281-288 (1989); Yamamori, Y. et al., Science 246:1412-1416 (1989), both of which references are incorporated herein by reference). Since the gene encoding lif has been cloned (Gough, N.M. et al., Reprod. Fertil. Dev. 1:281-288 (1989)), it is especially preferred to transform stomal cells with this gene, by means known in the art, and to then culture the ES cells on transformed stomal cells that secrete lif into the culture medium.

ES cell lines may be derived or isolated from any species (for example, chicken, etc.), although cells derived or isolated from mammals such as rodents (i.e. mouse, rat, hamster, etc.), rabbits, sheep, goats, fish, pigs, cattle, primates and humans are preferred.

#### V. The Production of Chimeric and Transgenic Plants

The chimeric or transgenic plants of the invention are produced through the regeneration of a plant cell which has received a DNA molecule through the use of the methods disclosed herein.

All plants from which protoplasts can be isolated and cultured to give whole regenerated plants can be transformed by the present invention so that whole plants are recovered which contain the introduced gene sequence. Some suitable plants include, for example, species from the genera Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manicot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersion, Nicotiana, Solanum, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Hemerocallis,

1 Nemesia, Pelargonium, Panicum, Pennisetum, Ranunculus,  
2 Senecio, Salpiglossis, Cucumis, Browallia, Glycine, Lolium,  
3 Zea, Triticum, Sorghum, Ipomoea, Passiflora, Cyclamen,  
4 Malus, Prunus, Rosa, Rubus, Populus, Santalum, Allium,  
5 Lilium, Narcissus, Ananas, Arachis, Phaseolus, Pisum and  
6 Datura.

7       There is an increasing body of evidence that practically  
8 all plants can be regenerated from cultured cells or  
9 tissues, including but not limited to all major cereal crop  
10 species, sugarcane, sugar beet, cotton, fruit and other  
11 trees, legumes and vegetables.

12       Plant regeneration from cultural protoplasts is  
13 described in Evans et al., "Protoplast Isolation and  
14 Culture," in Handbook of Plant Cell Culture 1:124-176  
15 (MacMillan Publishing Co., New York, 1983); M.R. Davey,  
16 "Recent Developments in the Culture and Regeneration of  
17 Plant Protoplasts," Protoplasts, 1983 - Lecture Proceedings,  
18 pp. 19-29 (Birkhauser, Basel, 1983); P.J. Dale, "Protoplast  
19 Culture and Plant Regeneration of Cereals and Other  
20 Recalcitrant Crops," in Protoplasts 1983 - Lecture  
21 Proceedings, pp. 31-41 (Birkhauser, Basel, 1983); and H.  
22 Binding, "Regeneration of Plants," in Plant Protoplasts, pp.  
23 21-37 (CRC Press, Boca Raton, 1985).

24       Regeneration varies from species to species of plants,  
25 but generally a suspension of transformed protoplasts  
26 containing the introduced gene sequence is formed. Embryo  
27 formation can then be induced from the protoplast  
28 suspensions, to the stage of ripening and germination as  
29 natural embryos. The culture media will generally contain  
30 various amino acids and hormones, such as auxin and  
31 cytokinins. It is also advantageous to add glutamic acid  
32 and proline to the medium, especially for such species as  
33 corn and alfalfa. Shoots and roots normally develop

1 simultaneously. Efficient regeneration will depend on the  
2 medium, on the genotype, and on the history of the culture.  
3 If these three variables are controlled, then regeneration  
4 is fully reproducible and repeatable.

5 The mature plants, grown from the transformed plant  
6 cells, are selfed to produce an inbred plant. The inbred  
7 plant produces seed containing the introduced gene sequence.  
8 These seeds can be grown to produce plants that express this  
9 desired gene sequence.

10 Parts obtained from the regenerated plant, such as  
11 flowers, seeds, leaves, branches, fruit, and the like are  
12 covered by the invention. Progeny and variants, and mutants  
13 of the regenerated plants are also included within the scope  
14 of this invention.

15 As used herein, variant describes phenotypic changes  
16 that are stable and heritable, including heritable variation  
17 that is sexually transmitted to progeny of plants.

## 18 VI. GENE EXPRESSION

19  
20 In one embodiment, the DNA molecule(s) which are to be  
21 introduced into the recipient cells in accordance with the  
22 methods of the present invention will be incorporated into  
23 a plasmid or viral vector (or a derivative thereof) capable  
24 of autonomous replication in a host cell.

25 Preferred prokaryotic vectors include plasmids such as  
26 those capable of replication in E. coli such as, for  
27 example, pBR322, ColE1, pSC101, pACYC 184,  $\pi$ VX. Such  
28 plasmids are, for example, disclosed by Maniatis, T., et al.  
29 (In: Molecular Cloning, A Laboratory Manual, Cold Spring  
30 Harbor Press, Cold Spring Harbor, NY (1982)). Bacillus  
31 plasmids include pC194, pC221, pT127, etc. Such plasmids  
32 are disclosed by Gryczan, T. (In: The Molecular Biology of  
33

-46-

1 the Bacilli, Academic Press, NY (1982), pp. 307-329).  
2 suitable Streptomyces plasmids include pIJ101 (Kendall,  
3 K.J., et al., J. Bacteriol. 169:4177-4183 (1987)), and  
4 Streptomyces bacteriophages such as  $\phi$ C31 (Chater, K.F., et  
5 al., In: Sixth International Symposium on Actinomycetales  
6 Biology, Akademiai Kiado, Budapest, Hungary (1986), pp. 45-  
7 54). Pseudomonas plasmids are reviewed by John, J.F., et  
8 al. (Rev. Infect. Dis. 8:693-704 (1986)), and Izaki, K.  
9 (Jpn. J. Bacteriol. 33:729-742 (1978)).

10 Examples of suitable yeast vectors include the yeast 2-  
11 micron circle, the expression plasmids YEP13, YCP and YRP,  
12 etc., or their derivatives. Such plasmids are well known in  
13 the art (Botstein, D., et al., Miami Wntr. Symp. 19:265-274  
14 (1982); Broach, J.R., In: The Molecular Biology of the  
15 Yeast Saccharomyces: Life Cycle and Inheritance, Cold  
16 Spring Harbor Laboratory, Cold Spring Harbor, NY, p. 445-470  
17 (1981); Broach, J.R., Cell 28:203-204 (1982)).

18 Examples of vectors which may be used to replicate the  
19 DNA molecules in a mammalian host include animal viruses  
20 such as bovine papilloma virus, polyoma virus, adenovirus,  
21 or SV40 virus.

## 22 VII. Uses of the Present Invention

23 The methods of the present invention permit the  
24 introduction of a desired gene sequence into an animal or  
25 plant cell.

26 In a first embodiment, the methods of the present  
27 invention may be used to introduce DNA into germ line cells  
28 of animals in order to produce chimeric or transgenic  
29 animals which contain a desired gene sequence. The animals  
30 which may be produced through application of the described  
31 method include chicken, non-human mammals (especially,  
32  
33



1 rodents (i. . mous , rat, hamst r, tc.), rabbits, sheep,  
2 goats, fish, pigs, cattl and non-human primates).

3 As stated above, the desired g n s quence may be of any  
4 length, and have any nucleotide sequence. In particular, it  
5 is possible to design the sequence of the desired gene  
6 sequence in order to create single, or multiple base  
7 alterations, insertions or deletions in any preselected gene  
8 of a cell.

9 If such changes are within a translated region of a  
10 native gene sequence, then a new protein variant of a nativ  
11 protein can be obtained. Such a procedure can, for example  
12 be used to produce animals which produce improved (i.e. more  
13 stable, more active, etc.) enzymes, binding proteins,  
14 receptors, receptor ligands, etc.

15 The methods of the present invention may be used to  
16 produce cells in which a natural gene has been replaced with  
17 a heterologous gene. A gene is said to be heterologous to  
18 a transgenic cell if it is derivable from a species other  
19 than that of the transgenic cell.

20 In one embodiment, this replacement may be accomplish d  
21 in a single step (Figure 3). To accomplish such  
22 replacement, a DNA molecule containing a desired gene  
23 sequence and a region of homology is introduced into a  
24 recipient cell. A selectable marker gene is also introduc d  
25 into the cell, and used to select for cells which have  
26 underwent recombination. The method results in the  
27 replacement of the normal sequences adjacent to the region  
28 of homology with the heterologous sequences of the desired  
29 DNA sequence.

30 In a second embodiment, this replacement may be  
31 accomplished in a two steps (Figure 4). As in the  
32 embodiment described above, a cell is provided with a DNA  
33 m l cul containing a desired gene sequence and a r gion of

-48-

1 homology. Th DNA molecule also contains a selectable  
2 marker gene used to select for cells which have undergone a  
3 recombinational event that has resulted in the insertion of  
4 the introduced DNA molecule into their chromosomes at the  
5 site of homology. The structure of such an insertion site  
6 is depicted in Figure 4A.

7 Significantly, in this embodiment, the introduced DNA  
8 molecule will also contain a "negative selectable" marker  
9 gene which can be used to select for cells which undergo a  
10 second recombinational event that results in the loss of the  
11 inserted DNA.

12 As shown in Figure 4B, a second DNA molecule is employed  
13 to complete the gene replacement. This second DNA molecule  
14 need not contain any selectable marker gene. Upon receipt  
15 of the second DNA molecule, a second recombinational event  
16 occurs which exchanges the "second" DNA molecule for th  
17 integrated "first" DNA molecule (including the desired DNA  
18 sequence, the selectable marker sequence, and the "negativ  
19 selectable" marker sequence contained on that molecule).  
20 This aspect of the invention is illustrated in Figure 4B.

21 In another embodiment of the invention, subtle mutations  
22 may be introduced into a desired locus using a "cassette"  
23 construct containing both a positive selection marker (such  
24 as the np<sup>t</sup>II gene or the gpt gene) and a negative selection  
25 marker (such as the tk gene). In this embodiment, one first  
26 uses the positive selection capacity of the construct t  
27 introduce the two selection markers into a desired locus.  
28 One then introduces the desired subtle mutations (substi-  
29 tutions, insertions, deletions, etc.) by providing a cell  
30 with a DNA molecule that contains the desired mutation. By  
31 selecting for the loss of the "cassette" (using the negativ  
32 selection marker), one can select for recombinational events  
33 which result in the replacement of the "cassette" sequence

1 with the DNA sequence containing the desired mutation. This  
2 embodiment of the invention is illustrated in Figure 5.

3 The methods of the present invention may also be used to  
4 replace contiguous regions of a chromosome with any desired  
5 gene sequence. Thus, the present invention is not limited  
6 in the size of the DNA regions which may be altered or  
7 replaced. This aspect of the present invention is  
8 illustrated in Figure 6, as a series of 5 steps (Figures 6A-  
9 6E). The method is applicable to any gene sequence. It is  
10 especially useful in producing cells which contain  
11 heterologous immunoglobulins (such as the heavy chain locus  
12 of an immunoglobulin).

13 The first step in replacing a large region of a  
14 chromosome with a desired sequence involves setting up an  
15 initial target. In this step, a recipient cell is provided  
16 with a DNA molecule which contains a "first fragment" of the  
17 total desired replacement sequence (Figure 6A). This "first  
18 fragment" of the desired replacement sequence contains a  
19 selectable marker sequence (most preferably the nptII gene)  
20 at its end.

21 The DNA molecule also contains a "dual selection" gene  
22 sequence which encodes a non-functional fragment of a gene  
23 sequence for which both a positive and a negative selection  
24 exists. An example of such a gene is the gpt gene when used  
25 in the context of an hprt cell. Cells which express a  
26 functional gpt gene can be selected for by their ability to  
27 grow in HAT medium; Cells which lack a functional gpt gene  
28 can be selected for by their ability to grow in the presence  
29 of 6-thioguanine.

30 Homologous recombination results in the insertion of the  
31 DNA molecule into the cell's genome at the region of  
32 homology (Figure 6A). Importantly, since this step results  
33 in the creation of a cell whose genome contains the

1        s 1 ctabl marker g n , it is possibl to select for th  
2        desired recombinational event.

3        In the second step f th m thod, a s cond DNA molecule  
4        is provided to the cell. This second DNA molecule contains  
5        a "second fragment" of the desired replacement sequence as  
6        well as a sequence of the dual selection gene that, due to  
7        an internal deletion, is incapable of encoding a functional  
8        gene product. Homologous recombination results in the  
9        insertion of the second DNA molecule into the cell's genome  
10       in a manner so as to create a functional dual selection gene  
11       (Figure 6B). Recombination also results in the integration  
12       of a non-functional fragment of the dual selection gene.  
13       Importantly, since this step results in the creation of a  
14       cell whose genome contains a functional dual selection gene,  
15       it is possible to select for the desired recombinational  
16       event.

17       In the third step of the method, a third DNA molecule is  
18       provided to the cell. This third DNA molecule contains both  
19       the "first" and "second" fragments of the desired  
20       replacement sequence. Homologous recombination results in  
21       the insertion of the third DNA molecule into the cell's  
22       genome in a manner so as to delete the functional dual  
23       selection gene. The non-functional fragment of the dual  
24       selection gene (formed in step 2) is not affected by th  
25       recombination, and is retained (Figure 6C). Importantly,  
26       since this step results in the creation of a cell whose  
27       genome lacks the dual selection gene, it is possible to  
28       select for the desired recombinational event.

29       In the fourth step of the method, a fourth DNA molecu  
30       is provided to the cell. This fourth DNA molecule contains  
31       a "third fragment" of the desired replacement sequence as  
32       well as a sequence of the dual selection gene that, as in  
33       step 2, is incapable of encoding a functional gene product

-51-

1 due to an internal deletion. Homologous recombination  
2 results in the insertion of the fourth DNA molecule into the  
3 cell's genome in a manner so as to create a functional dual  
4 selection gene (Figure 6D). Recombination also results in  
5 the integration of a non-functional fragment of the dual  
6 selection gene. Importantly, since this step results in the  
7 creation of a cell whose genome contains a functional dual  
8 selection gene, it is possible to select for the desired  
9 recombinational event.

10 In the fifth step of the method, a fifth DNA molecule is  
11 provided to the cell. This fifth DNA molecule contains both  
12 the "second" and "third" fragments of the desired  
13 replacement sequence. Homologous recombination results in  
14 the insertion of the fifth DNA molecule into the cell's  
15 genome in a manner so as to delete the functional dual  
16 selection gene. The non-functional fragment of the dual  
17 selection gene (formed in step 4) is not affected by the  
18 recombination, and is retained (Figure 6C). Importantly,  
19 since this step results in the creation of a cell whose  
20 genome lacks the dual selection gene, it is possible to  
21 select for the desired recombinational event.

22 As will be appreciated, the net effect of the above-  
23 described steps is to produce a cell whose genome has been  
24 engineered to contain a "first," "second," and "third"  
25 "fragment" of a particular desired gene in a contiguous  
26 manner. The steps may be repeated as desired in order to  
27 introduce additional "fragments" into the cell's genome. In  
28 this manner, cells can be constructed which contain  
29 heterologous genes, chromosome fragments, or chromosomes,  
30 that could not be introduced using a single vector. As  
31 indicated above, each step of the method can be selected  
32 for.

1           In particular, this aspect of the present invention may  
2 be used to produce "humanized" antibodies (i.e., non-human  
3 antibodies which are non-immunogenic in a human) (Rabinson,  
4 R.R. et al., International Patent Publication  
5 PCT/US86/02269; Akira, K. et al., European Patent  
6 Application 184,187; Taniguchi, M., European Patent  
7 Application 171,496; Morrison, S.L. et al., European Patent  
8 Application 173,494; Neuberger, M.S. et al., PCT Application  
9 WO 86/01533; Cabilly, S. et al., European Patent Application  
10 125,023; Better, M. et al., Science 240:1041-1043 (1988);  
11 Liu, A.Y. et al., Proc. Natl. Acad. Sci. USA 84:3439-3443  
12 (1987); Liu, A.Y. et al., J. Immunol. 139:3521-3526 (1987);  
13 Sun, L.K. et al., Proc. Natl. Acad. Sci. USA 84:214-218  
14 (1987); Nishimura, Y. et al., Canc. Res. 47:999-1005 (1987);  
15 Wood, C.R. et al., Nature 314:446-449 (1985)); Shaw et al.,  
16 J. Natl. Cancer Inst. 80:1553-1559 (1988).

17           The method may also be used to produce animals having  
18 superior resistance to disease, animals which constitute or  
19 produce improved food sources, animals which provide fibers,  
20 hides, etc. having more desirable characteristics. The  
21 method may also be used to produce new animal models for  
22 human genetic diseases. For example, the method may be used  
23 to "humanize" the CD4 analog of an animal, and thus provide  
24 an animal model for AIDS. Such animal models can be used  
25 for drug testing, and thus hasten the development of new  
26 therapies for genetic diseases.

27           In addition, the present invention permits the formation  
28 of cells and of transgenic animals which contain mutations  
29 in medically or clinically significant heterologous genes.  
30 A gene is said to be medically or clinically significant if  
31 it expresses an isotype of a protein associated with a human  
32 or animal disease or condition. Examples of such genes  
33 include the genes which encode: topoisomerase p180, 5- $\alpha$

1 r ductase, ACAT, 5-lipoxygenase, the insulin receptor, the  
2 interleukin-2 receptor, the epidermal growth factor  
3 receptor, the serotonin receptor, the dopamine receptor, the  
4 GABA receptor, the  $V_2$  vasopressin receptors, G proteins  
5 (signal transduction), phospholipase C proteins, and  
6 insulin. A transgenic mouse produced by microinjection  
7 which expresses human insulin was reported by Selden, R.F.  
8 et al. (European Patent Publication No. 247,494, which  
9 reference is incorporated herein by reference).

10 The transgenic cells and animals discussed above can be  
11 used to study human gene regulation. For example,  
12 transgenic animals which express a human isotype of  
13 topoisomerase p180, 5- $\alpha$  reductase, ACAT, 5-lipoxygenase, or  
14 hormone or cytokine receptors would have utility in in vivo  
15 drug screening. The expression of topoisomerase p180 is  
16 associated with resistance to chemotherapeutics. Thus,  
17 agents which interfere with this enzyme could be used to  
18 enhance the effectiveness of chemotherapy. An animal,  
19 especially a rat, capable of expressing a human isotype of  
20 5- $\alpha$  reductase (especially in the prostate gland) would be  
21 highly desirable. ACAT is a key enzyme in lipid metabolism;  
22 an animal model for its regulation would be extremely  
23 valuable. Animals that express 5-lipoxygenase could be of  
24 interest to many research programs, particularly to screen  
25 isotype selective inhibitors. An animal which expressed  
26 human hormone or cytokine receptor proteins would be  
27 valuable in identifying agonists and antagonists of receptor  
28 action. Similarly, an animal that expressed components of  
29 the human signal transduction system (i.e. G proteins and  
30 phospholipase Cs, etc.) could be used to study the  
31 pathophysiologic consequences of disordered function of  
32 these proteins.

1       The present invention can be used to produce cells and  
2 animals which express human isotypes of transport proteins  
3 (i.e. proteins which facilitate or enable the transport of  
4 other molecules or ions across membranes in the gut, blood  
5 brain barrier, kidney, etc.). Such cells or animals can  
6 then be used to study the role of such proteins in  
7 metabolism. In particular, the extent and patterns of  
8 conjugation mediated by such isotypes may be studied in  
9 order to investigate the pharmacokinetic consequences of  
10 specific differences in protein structure or sequence.  
11 Glucoronide transferase, glycine conjugation and sulfation,  
12 methylases, and glutathione conjugation are examples of  
13 enzymes of particular interest in this regard.

14       The clearance of many compounds is mediated by  
15 esterases. Cells or animals which express heterologous  
16 isotypes of such esterases may be exploited in investigating  
17 such clearance.

18       Cells or animals which express isotypes of proteins  
19 involved in azo or nitro reduction would be desirable for  
20 research on the processes of azo or nitro reduction.

21       Significantly, potential therapeutic agents are  
22 frequently found to induce toxic effects in one animal model  
23 but not in another animal model. To resolve the potential  
24 of such agents, it is often necessary to determine the  
25 metabolic patterns in various species, and to then determine  
26 the toxicities of the metabolites. The present invention  
27 permits one to produce transgenic cells or animals which  
28 could facilitate such determinations.

29       The methods of the present invention may be used to  
30 produce alterations in a regulatory region for a native gene  
31 sequence. Thus, the invention provides a means for altering  
32 the nature or control of transcription or translation of any  
33 native gene sequence which is regulated by the regulatory



-55-

1 r g i n. F r exampl , it is possibl t introduc mutations  
2 which rem v f edback inhibiti n, and thus r sult in  
3 increased gene expression. Similarly, it is possible to  
4 impair the transcriptional capacity of a sequence in order  
5 to decrease gene expression. Such alterations ar  
6 especially valuable in gene therapy protocols, and in the  
7 development of improved animal models of human disease. For  
8 example, the capacity to increase insulin gene transcription  
9 or translation provides a potential genetic therapy for  
10 diabetes. Similarly, the ability to impair the synthesis of  
11 beta globin chains provides an animal model for beta-  
12 thalassemia.

13 The methods of the present invention, quite apart from  
14 their uses in veterinary and human medicine, may be used to  
15 investigate gene regulation, expression and organization in  
16 animals.

17 Since the methods of the present invention utiliz  
18 processes of DNA repair and recombination, agents which  
19 inhibit or impair the present methods may act by affecting  
20 these processes. Since agents which impair DNA repair and  
21 recombination have potential antineoplastic utility, th  
22 present invention provides a means for identifying novel  
23 antineoplastic agents.

24 The present invention may additionally be used to  
25 facilitate both the cloning of gene sequences, and the  
26 mapping of chromosomes or chromosomal abnormalities.

27 Since the desired gene sequence need not be homologous  
28 or analogous to any native gene sequence of the recipient  
29 cell, the methods of the present invention permit one to  
30 produce animals which contain and express foreign gen  
31 sequences. If the cell expresses an analogous gene, the  
32 desired gene sequence may be expressed in addition to such  
33 analogous cellular gen s (for exampl , an animal may express

1 both a "humanized" receptor and an analogous native  
2 receptor). Thus, for example, the invention provides a  
3 means for producing animals which express important human  
4 proteins (such as human interferons, tissue plasminogen  
5 activator, hormones (such as insulin and growth hormone),  
6 blood factors (such as Factor VIII), etc.).

7 In a second embodiment, the methods of the invention may  
8 be used to introduce DNA into plant cells which can then be  
9 manipulated in order to produce chimeric or transgenic  
10 plants. The plants which may be produced through  
11 application of the disclosed method include all  
12 multicellular, higher (i.e. non-fungal) plants. A non-  
13 fungal plant is any plant which is not a fungus or yeast.

14 In a third embodiment, the methods of the invention may  
15 be used to introduce DNA into the somatic cells of an animal  
16 (particularly mammals including humans) or plant in order to  
17 provide a treatment for genetic disease (i.e. "gene  
18 therapy"). The principles of gene therapy are disclosed by  
19 Oldham, R.K. (In: Principles of Biotherapy, Raven Press, NY,  
20 1987), and similar texts.

21 In this third embodiment, the genetic lesion which  
22 causes the disease is replaced with a gene sequence encoding  
23 a preferred gene product. Examples of such genetic lesions  
24 are those responsible for diseases such as cystic fibrosis,  
25 phenylketonuria, hemophilia, von Willebrand's Disease,  
26 sickle cell anemia, thalassemia, galactosemia, fructose  
27 intolerance, diseases of glycogen storage, hyp r-  
28 cholesterolemia, juvenile diabetes, hypothyroidism,  
29 Alzheimer's Disease, Huntington's Disease, Gout, Lesch-Nyhan  
30 Syndrome, etc. (Bondy, P.K. et al., In: Disorders of  
31 Carbohydrate Metabolism, pp 221-340, Saunders (1974);  
32 Coleman, J. et al., Molecular Mechanisms of Disease, Yale  
33 University Press, (1975)). Disclosures of the methods and

us s for gene therapy ar provid d by Boggs, S.S. (Int. J. Cell Clon. 8:80-96 (1990)); Karson, E.M. (Biol. Reprod. 42:39-49 (1990)); Ledley, F.D., In: Biotechnology, A Comprehensive Treatise, volume 7B, Gene Technology, VCH Publishers, Inc. NY, pp 399-458 (1989)); all of which references are incorporated herein by reference.

In a fourth embodiment, the methods of the invention may be used to provide a treatment to protect recipient animals or plants from exposure to viruses, insects or herbicides (in the case of plants), insecticides, toxins, etc. In this embodiment, the introduced gene would provide the recipient with gene sequences capable of mediating either an enhanced or novel expression of an enzyme, or other protein, capable of, for example, degrading an herbicide or toxin. For example, a plant cell may receive a gene sequence capable of mediating an enhanced or novel expression of a chitinase, thus conferring increased resistance to insect parasites.

When providing the desired gene sequence to the cells of an animal, pharmaceutically acceptable carriers (i.e. liposomes, etc.) are preferably employed. Such gene sequences can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined in admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, are described, for example, in Nicolau, C. et al. (Crit. Rev. Ther. Drug Carrier Syst. 6:239-271 (1989)), which reference is incorporated herein by reference.

In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the desired gene sequence together with a suitable amount of carrier vehicle.

1 Additional pharmac utical m thods may be employed to  
2 c ntrol th durati n f acti n. Control r lease  
3 preparati ns may be achieved thr ugh th use of polymers t  
4 complex or absorb the desired gene sequence (either with r  
5 without any associated carrier). The controlled delivery  
6 may be exercised by selecting appropriate macromolecul s  
7 (for example polyesters, polyamino acids, polyvinyl,  
8 pyrrolidone, ethylenevinylacetate, methylcellulos ,  
9 carboxymethylcellulose, or protamine, sulfate) and the  
10 concentration of macromolecules as well as the methods f  
11 incorporation in order to control release. Another possible  
12 method to control the duration of action by controlled  
13 release preparations is to incorporate the agent into  
14 particles of a polymeric material such as polyesters,  
15 polyamino acids, hydrogels, poly(lactic acid) or ethyl ne  
16 vinylacetate copolymers. Alternatively, instead of  
17 incorporating these agents into polymeric particles, it is  
18 possible to entrap these materials in microcapsules  
19 prepared, for example, by coacervation techniques or by  
20 interfacial polymerization, for example, hydroxymethylcellu-  
21 lose or gelatine-microcapsules and poly(methylmethacylat )  
22 microcapsules, respectively, or in colloidal drug delivery  
23 systems, for example, liposomes, albumin microspheres,  
24 microemulsions, nanoparticles, and nanocapsules or in  
25 macroemulsions.

26 In a fifth embodiment, the methods of the present  
27 invention may be used to improve the food or fiber  
28 characteristics of plants or non-human animals. For  
29 example, the methods can be used to increase the overall  
30 levels of protein synthesis thereby resulting in fast r  
31 growing plants or non-human animals, or in the production of  
32 plants and non-human animals which have increased food  
33 value.

## EXAMPLE 1

### ELECTROPORATION

DNA Preparation:

The large-scale digest was then extracted once with an equal volume of phenol/chloroform and once with an equal volume of chloroform. The DNA was precipitated with 2.4 volumes of ethanol, pelleted by centrifugation, and dried using a Speed-Vac.

The pelleted DNA was then resuspended at the desired concentration (usually 1  $\mu\text{g}/\mu\text{l}$ ) in a sterile Tris-EDTA buffer such as 0.1X TE (25  $\mu\text{l}$  of DNA per electroporation). The concentration of the DNA was then measured with a fluorometer.

### Preparation of Cells for Electroporation:

Embryonic stem cells of the AB1 cell line were cultured to approximately 80% confluence according to the methods of E.J. Robertson (In: Teratocarcinomas and Embryonic Stem Cells: A Practical Approach, (E.J. Robertson, Ed.), IRL Press, Oxford, 1987, pp 71-112). Cells were cultured in the presence of stomal cells which expressed l1f into the culture medium. Cells were passaged 1:2 the day before electroporation, and fed 4 hours before harvesting.

Cells were harvested by trypsinizing the cells, and by resuspending in media (cells from 2 x 10 cm plates were combined in a total volume of 10 ml in a 15 ml tube).

The cells were pelleted by centrifugation, and the supernatant was removed by aspiration. The cells were then resuspended in 10 ml of phosphate buffered saline and the total number of cells was determined by counting a 20  $\mu$ l aliquot. The usual yield is  $30 \times 10^6$  cells per 10 cm plate.

The cells were then pelleted by centrifugation and the supernatant was removed by aspiration. Cells were resuspended at a density of  $11 \times 10^6$  cells/ml. A 20  $\mu$ l aliquot was counted to confirm this cell density.

### Electroporation

Cells, prepared as described above, were incubated in the presence of an appropriate amount of DNA in a 15 ml tube. 25  $\mu$ l of DNA and 0.9 ml of cells were used for each electroporation.

The mixture was allowed to incubate at room temperature for 5 minutes (this step may, however, be omitted).

The cell/DNA mixture was then carefully aliquoted into electroporation cuvettes (0.9 ml per cuvette; the volume is

important). The cuvette was placed in the electroporation holder with the filter electrodes in contact with the metal holding clips.

Electroporation was accomplished using a Biorad GenePulser set at 230V, 500  $\mu$ F (this requires a capacitance extender). The time constant should read between 5.6 and 7.0.

The cuvette was left at room temperature for 5 minutes and then the cells were plated at an appropriate density (up to  $2 \times 10^7$  cells/100 mm plate or  $6 \times 10^6$  cells/60 mm plate). When G418 was used as a selective agent, this cell density should not be exceeded since G418 takes 3-4 days before killing starts and plates will become over-confluent. When G418 selection was to be applied, it is applied 24 hours post-electroporation. G418 concentration must be titrated for every batch.

The plate(s) were re-fed with fresh media + G418 every day for the first 6-7 days (until colonies are visible and most cell debris has been removed). If using FIAU (0.2  $\mu$ M) selection, this may proceed simultaneously.

The typical yield for RV4.0 (Thomas, K.R. *et al.*, *Cell* 51:503-512 (1987)) is up to  $10^4$  colonies/ $10^7$  cells/100 mm plate. Although this yield may be significantly (and unpredictably) different from the yield obtained when other constructs are used, the use of the method always results in the recovery of some colonies of cells which contain the electroporated DNA.

Colonies may be picked as early as 8 days. It is most preferred to pick colonies at around 10-11 days. Colonies may, however, be recovered up to 18-21 days after the electroporation.

-62-

**EXAMPLE 2**  
**CO-ELECTROPORATION OF ES CELLS**

To illustrate the invention, embryonic stem ("ES") cells were co-electroporated with a 4.5 kb nptII-containing vector (pPGKneobpA) which had been linearized by treatment with XhoI restriction endonuclease, and with the 6.5 kb HPRT vector, AD 8 (linearized with SacI) (Figure 7). Electroporation (230 V, 500  $\mu$ F) were done on 0.9 ml aliquot of CCEp24 cells ( $7.5 \times 10^6$  cells/ml).

The electroporation reactions were conducted using molar ratios of 1:1, 1:10, and 1:100 (nptII DNA:HPRT DNA). The total amount of DNA provided was either 25, 50, 100, or 200  $\mu$ g. The vectors used in this experiment are illustrated in Figure 7. The results of this experiment are shown in Table 1.

TABLE 1  
CO-ELECTROPORATION OF nptII AND hpri GENE SEQUENCES

Average of Number of Colonies Formed per  $1 \times 10^6$  Cells  
( $\mu$ g of DNA (# = Number of trials averaged))

Ratio	200			100			50			25		
of DNA	G418 <sup>R</sup>	TG <sup>R</sup>	#	G418 <sup>R</sup>	TG <sup>R</sup>	#	G418 <sup>R</sup>	TG <sup>R</sup>	#	G418 <sup>R</sup>	TG <sup>R</sup>	#
1:1	233	2.7	3	101	1.5	3	64	0	3	23	0	5
1:10	46	0	3	16	0	5	8.7	0	7	nd	nd	
1:100	8	0.2	5	4.3	0	7	1.6	0	7	nd	nd	

This experiment shows that co-electroporation of an hpri gene sequence with an nptII-containing gene sequence in the presence of selection for only the nptII-containing sequence, resulted in recombination of both the nptII and hpri DNA molecules.



-63-

The frequencies of recombination are shown in Table 2 below.

TABLE 2  
FREQUENCY OF RECOMBINATION

Expt	Ratio Neo:Hprt	[DNA] μg/ml	G418 <sup>R</sup> /10 <sup>5</sup>	TG <sup>R</sup> /10 <sup>7</sup>	TG <sup>R</sup> /G418 <sup>R</sup>
A	1:1	200	23.3	2.6	1/873
B	1:1	100	10.1	1.0	1/1010
C	1:100	200	0.8	0.2	1/400*
Cont	---	25	10.8	2.7	1/402

The reactions were carried out as described above. The reproducibility of the experimental results was examined. The results of this experiment are shown in Table 3.

TABLE 3  
EFFECT OF MODIFIED CO-ELECTROPORATION PROTOCOL ON  
RECOMBINATION FREQUENCY

Molar ratios Neo:	DNA per zap (μg)	# of zap	G418 <sup>R</sup> /HPRT- colonies (total)	HPRT- G418R	HPRT- (per cell transfected) (X 10 <sup>-9</sup> )	G418 <sup>R</sup> (X 10 <sup>-6</sup> )
1:1	200	8	16,150 / 32	1/504	400	202
	100	3	3,030 / 3	1/1,010	100	105
	50	3	1,920 / 0		67	
	25	5	1,150 / 0		24	
1:10	200	16	608 / 7	1/868	43	47
	100	5	800 / 0		17	
	50	7	609 / 0		9	
1:100	200	5	400 / 1	1/400	8	
	100	7	300 / 0		4.5	
	50	7	112 / 0		1.7	

**EXAMPLE 3**  
**HOMOLOGOUS RECOMBINATION**

In order to investigate the chromosomal structure which is produced by the recombination of the vectors of the above-described vectors into the chromosomes of recipient cells, the following experiments were conducted.

For this purpose, a vector was used which contained a 6.5 kb region of homology with the cellular hprt locus. The vector also contained the nptII gene, as a selectable marker. The vector was linearized with XhoI and provided to ES cells by electroporation, as described above. Cells which became resistant to G418 were selected and their DNA was analyzed to determine if it contained restriction fragments that were consistent with the predicted integration structure.

The vector used, and the predicted integration structure are illustrated in Figure 8. Gel electrophoresis of restriction digests of cellular DNA confirmed that the G418 resistant cells contained the hprt structure shown in Figure 8. This finding confirmed that the vector had integrated into the chromosome of the cell by homologous recombination at the hprt locus.

**EXAMPLE 4**  
**REVERSION OF RECOMBINANTS**

The effect of the size of the region of homology carried by the vector on the reversion frequency of recombinants was determined. Recombinants containing a vector having 6.8 kb of homology with the hprt locus were prepared as described in Example 3. Using the same method, recombinants were also prepared which contained a similar vector having only 1.3 kb

of homology with the hprt locus. The structures of the insertion site of the 6.8 kb vector is illustrated in Figure 8. The reversion frequency of the two constructs is shown in Table 4. The structure obtained from the reversion of the insertion is shown in Figure 9.

TABLE 4  
REVERSION FREQUENCY

<u>Duplication</u>	<u># Clones</u>	<u># Revertible</u>	<u>Frequency x10<sup>-5</sup></u>
6.8 kb	19	19	3.3 to 0.2
1.3 kb	2	2	1.2 to 0.3

EXAMPLE 5  
TARGETING FREQUENCY OF INSERTION AND REPLACEMENT VECTORS

A series of different vectors were used to investigate the targeting frequency achieved through the use of the methods of the invention. These vectors contained 6.8 kb of homology with the murine hprt gene and had regions of heterology either at the linearization site or internally (Figure 2).

For this purpose,  $10^8$  cells were electroporated into ES cells, prepared as described above, and plated onto 10 x 90 mm plates. After 24 hours G418 (at 350  $\mu$ g/ml) was added to the media. After 5 days selection  $10^{-5}$  M 6-thioguanine was added to 9 plates, 1 was retained under G418 selection as the transfection control. Selection was continued for an additional 7 days. Colonies were scored at this time and expanded for southern analysis as separate clones. Targeting efficiencies are detailed for each of the vectors (Figure 2; Table 5).

-66-

Southern analysis showed that the majority of the 6-TG<sup>R</sup> clones had the predicted integration structure depicted for HindIII digestion in Figure 8.

Reversion of the hprt clones was done by measuring HAT<sup>R</sup>. Cells were clonally expanded under 6-TG selection to prevent "jackpot" effects caused by the early recombinational loss of the duplicated element giving rise to a large number of colonies by cell division. When 10<sup>7</sup> cells were obtained, the cells were resseeded onto 90 mm plates without selection for 48 hours. After 48 hours HAT selection was applied and resistant colonies were scored 10 days later, typically 20 to 200 colonies were observed per 10<sup>7</sup> cells plated (Table 4). Every clone examined reverted at a similar frequency.

TABLE 5  
REPLACEMENT AND INSERTION VECTORS: TARGETING AND FREQUENCY

<u>Gene</u>	<u>Homology</u>	<u>Vector</u>	<u>Frequency</u>	
Hprt	6.8 kb	RV	1/300	10X
Hprt	6.8 kb	IV	1/32	
Hprt	1.3 kb	RV	<1/5000	12X
Hprt	minimum			
Hprt	6.8 kb	IV	1/400	
Hox2.6	3.2 kb	IV+	1/33	

RV=Replacement Vector; IV=Insertion Vector

#### EXAMPLE 6 SELECTION FOR HOMOLOGOUS RECOMBINATION

It is possible to use "Poly A Selection" in order to enhance the selection of cells which have integrated the introduced DNA by homologous recombination.

1           If an introduced DNA molecule were to integrate at  
2 random into the host chromosome, it would generally not  
3 integrate at a site adjacent to a necessary 3'  
4 polyadenylation site. Thus, the mRNA produced by the  
5 transcription of such randomly inserted constructs would  
6 generally lack polyadenylation. This fact can be exploited  
7 by using vectors which permit one to select for a  
8 recombinational event that results in integration adjacent  
9 to the natural polyadenylation site of the introduced gene  
10 sequence (i.e. by homologous recombination rather than by  
11 random insertion).

12           To illustrate this aspect of the invention, three  
13 vectors were constructed which contain fragments of the hprt  
14 gene (Figure 10). As shown in Figure 10, the vectors  
15 contain exons 7, 8, and 9 of the hprt gene. The  
16 polyadenylation site is located in exon 9. A HindIII site  
17 is present within exon 9, and an EcoRI site is located after  
18 the end of the exon.

19           The first vector employed contained a 5.0 kb region, and  
20 thus contained the polyadenylation site of exon 9 (Vector 6,  
21 Figure 10). As shown in Table 6, the frequency of insertion  
22 was high (i.e. frequency of G418 resistant colonies was  $24 \times 10^{-5}$ ),  
23 but only 1/941 colonies showed the dual thioguanin  
24 resistance and G418 resistance which would characterize a  
25 desired recombinant (i.e. a recombinant in which integration  
26 had resulted in an intact hprt gene and an intact nptII  
27 gene). Thus, some random integration is occurring.

28           Similarly, when a vector of 3.5 kb was employed (Vector  
29 10) which contained DNA from the XbaI site to the EcoRI site  
30 of Vector 6, the frequency of insertion was high (i.e.  
31 frequency of G418 resistant colonies was  $21 \times 10^{-5}$ ), but only  
32 1/770 colonies showed the dual thioguanine resistance and  
33 G418 resistance which would characterize a desired

recombinant (Table 6). This finding demonstrates that some random integration is occurring.

If, however, a vector is employed which lacks the polyadenylation site of exon 9 (i.e. Vector 9), random integration does not result in expression of a functional nptII transcript. Thus, the frequency of G418 resistant colonies is low ( $1.4 \times 10^{-5}$ ). Since the number of colonies evidencing random integration is suppressed, the overall frequency of recovery of the desired recombinants is enhanced (i.e. an overall efficiency of 1/100 for the dual resistant colonies (Table 6). Thus, the poly A selection results in an approximate increase of overall efficiency of nearly 10 fold. Poly A selection may therefore be advantageously used in situations where one desires to minimize or avoid the screening of colonies to identify random versus homologous recombinants.

TABLE 6  
POLY A SELECTION

VECTOR #	SIZE (kb)	G418 <sup>R</sup> ( $\times 10^{-5}$ )	TG <sup>R</sup> ( $\times 10^{-7}$ )	TG <sup>R</sup> /G418 <sup>R</sup>
6	5.0	24	2.5	1/941
9	3.0	1.4	1.4	1/100
10	3.5	21	2.7	1/770

EXAMPLE 7  
INTRODUCTION OF SUBTLE MUTATIONS IN THE C-SRC LOCUS

The methods of the present invention were further illustrated by their use to produce cells having precise and subtle mutations in the c-src locus of ES cells. The c-src locus contains several exons, which are designated as "boxed" regions 2 and 3' in Figure 11. As shown in Figure

-69-

1 11A, the natural allele of exon 3' does not contain a  
2 HindIII site.

3 The sequence of a portion of exon 3' is shown in Figure  
4 11C. As shown in Figure 11C, a 9 bp insertion into this  
5 exon will result in the formation of a HindIII site.

6 To accomplish this change in the sequence of exon 3', a  
7 vector (src 14) was prepared. As shown in Figure 11B, the  
8 src 14 vector is homologous to a region of the c-src locus.  
9 The exon 3' sequence of the vector, however, has been  
10 altered to contain the 9 base pair insertion needed to  
11 create a HindIII site (Figure 11C).

12 The src 14 vector was introduced into ES cells by co-  
13 electroporation with a second vector (PGKneo) that contained  
14 the nptII gene, at a total DNA concentration of 25 µg/ml and  
15 a molar ratio of 1:5 (neo vector to targeting vector) in the  
16 manner described above.

17 Cells were cultured in the presence of G418 for 12 days  
18 in order to select for recombinant cells in which the nptII  
19 gene had integrated. These recombinant cells were then  
20 screened, using PCR, for cells which had undergone a  
21 recombinational event resulting in the replacement of the  
22 natural exon 3' locus with the HindIII site-containing exon  
23 3' sequence of the src 14 vector.

24 Southern analysis of the colonies identified by PCR  
25 screening using probes B and C (Figure 11B) demonstrated  
26 that the natural exon 3' locus had been altered, as desired,  
27 to contain a HindIII site. This experiment demonstrated  
28 that subtle insertions can be introduced into any cellular  
29 gene.

30 To further illustrate the capacity of the present  
31 invention to introduce complex, predetermined mutations into  
32 the genome of a recipient cell, exon 3' of the c-src gene of

-70-

1 an ES cell was mutated to contain two different substitution  
2 mutations.

3 As shown in Figure 12A, the natural allele of exon 3"  
4 does not contain either an NheI site or an EcoRI site. As  
5 shown in Figure 12C, however, the replacement of the natural  
6 sequence ACC TGG TTC of exon 3" with the sequence TAG CTA  
7 GCT will result in the formation of an NheI site.  
8 Similarly, replacement of ACA with GAA in exon 3" will  
9 create an EcoRI site (Figure 12C).

10 To accomplish these changes in the sequence of exon ",  
11 a vector (src 33) was prepared. As shown in Figure 12B, the  
12 src 33 vector is homologous to a region of the c-src locus.  
13 The exon 3" sequence of the vector, however, has been  
14 altered to contain the substitutions indicated above (Figure  
15 12C).

16 The src 33 vector was introduced into ES cells by  
17 electroporation, in concert with a second vector that  
18 contained the nptII gene, in the manner described above.  
19 Cells were cultured in the presence of G418 in order to  
20 select for recombinant cells in which the nptII gene had  
21 integrated. These recombinant cells were then screened,  
22 using PCR, for cells which had undergone a second  
23 recombinational event resulting in the replacement of the  
24 natural exon 3" locus with the exon 3" sequence of the src  
25 33 vector.

26 Southern analysis of the colonies identified by PCR  
27 screening using probes A and C (Figure 12C) demonstrated  
28 that the natural exon 3" locus had been altered, as desired,  
29 to contain both the NheI and the EcoRI sites. This  
30 experiment demonstrated that subtle substitutions can be  
31 introduced into any cellular gene.

32 While the invention has been described in connection  
33 with specific embodiments thereof, it will be understood



-71-

1       that it is capabl   of further modifications and this  
2       applicati n is intended to cover any variations, uses, or  
3       adaptations of the invention following, in general, the  
4       principles of the invention and including such departures  
5       from the present disclosure as come within known or  
6       customary practice within the art to which the invention  
7       pertains and as may be applied to the essential features  
8       hereinbefore set forth and as follows in the scope of the  
9       appended claims.

-72-

1  
2 WHAT IS CLAIMED IS:  
3

4 1. A method for obtaining a desired animal or non-fungal  
5 plant cell which contains a desired non-selectable gene  
6 sequence inserted within a predetermined gene sequence of  
7 said cell's genome, which method comprises:

8 A. incubating a precursor cell with a DNA molecule  
9 containing said desired non-selectable gene sequence,  
10 wherein said DNA molecule additionally contains two regions  
11 of homology which flank said desired gene sequence, and  
12 which are sufficient to permit said desired gene sequence to  
13 undergo homologous recombination with said predetermined  
14 gene sequence of said genome of said precursor cell;

15 B. causing said DNA molecule to be introduced into  
16 said precursor cell;

17 C. permitting said introduced DNA molecule to  
18 undergo homologous recombination with said predetermined  
19 gene sequence of said genome of said precursor cell to  
20 thereby produce said desired cell wherein said desired non-  
21 selectable gene sequence has been inserted into said  
22 predetermined gene sequence; and

23 D. recovering said desired cell.  
24

25 2. The method of claim 1 wherein said DNA molecule  
26 contains a detectable marker gene sequence.  
27

28 3. The method of claim 1 wherein said DNA molecule is  
29 introduced into said precursor cell by subjecting said  
30 precursor cell and said DNA molecule to electroporation.  
31

32 4. The method of claim 3 wherein in step B, said  
33 precursor cell is simultaneously subjected to

-73-

1 el ctroporati n with a second DNA molecul , said s cond DNA  
2 m l cule containing a detectable marker gene sequence.

3  
4 5. The method of claim 1 wherein said desired cell is a  
5 non-fungal plant cell.

6  
7 6. The method of claim 1 wherein said desired cell is an  
8 animal cell.

9  
10 7. The method of claim 6 wherein said animal cell is a  
11 somatic cell.

12  
13 8. The method of claim 7 wherein said animal cell is of  
14 an animal selected from the group consisting of a chicken,  
15 a mouse, a rat, a hamster, a rabbit, a sheep, a goat, a  
16 fish, a pig, a cow or bull, a non-human primate and a human.

17  
18 9. The method of claim 6 wherein said animal cell is a  
19 pluripotent cell.

20  
21 10. The method of claim 9 wherein said animal cell is of  
22 an animal selected from the group consisting of a chicken,  
23 a mouse, a rat, a hamster, a rabbit, a sheep, a goat, a  
24 fish, a pig, a cow or bull, and a non-human primate.

25  
26 11. The method of claim 9 wherein said pluripotent cell  
27 is an embryonic stem cell.  
28

12. The method of any one of claims 1-3 wherein said desired gene sequence is substantially homologous to said predetermined gene sequence of said precursor cell.

13. The method of claim 12 wherein said desired gene sequence is an analog of said predetermined sequence of said precursor cell.

14. The method of claim 12 wherein said desired gene sequence is a human analog of said predetermined sequence of said precursor cell.

15. The method of claim 12 wherein said desired cell is a non-human cell which expresses said desired gene sequence.

16. The method of claim 12 wherein said desired gene sequence encodes a protein selected from the group consisting of: a hormone, an immunoglobulin, a receptor molecule, a ligand of a receptor molecule, and an enzyme.

17. A non-fungal plant cell which contains an introduced recombinant DNA molecule containing a desired gene sequence, said desired gene sequence being flanked by regions of homology which are sufficient to permit said desired gene sequence to undergo homologous recombination with a predetermined gene sequence of the genome of said cell.

18. A non-human animal cell which contains an introduced recombinant DNA molecule containing a desired gene sequence, said desired gene sequence being flanked by regions of homology which are sufficient to permit said desired gene sequence to undergo homologous recombination with a predetermined gene sequence of the genome of said cell.

1  
2 19. The desired cell produced by the methods of any one  
3 of claims 1-3.

4  
5 20. The desired cell produced by the method of claim 11.

6  
7 21. The desired cell produced by the method of claim 12.

8  
9 22. A non-human animal containing a cell derived from the  
10 desired cell of claim 19, wherein said animal is either a  
11 chimeric or a transgenic animal.

12  
13 23. The non-human animal of claim 22, wherein said animal  
14 and said desired cell are of the same species, and wherein  
15 said species is selected from the group consisting of: a  
16 chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a  
17 goat, a fish, a pig, a cow or bull, and a non-human primate.

18  
19 24. A non-human animal containing a cell derived from the  
20 desired cell of claim 20, wherein said animal is either a  
21 chimeric or a transgenic animal.

22  
23 25. The non-human animal of claim 24, wherein said animal  
24 and said desired cell are of the same species, and wherein  
25 said species is selected from the group consisting of: a  
26 chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a  
27 goat, a fish, a pig, a cow or bull, and a non-human primate.

28  
29 26. A non-human animal containing a cell derived from the  
30 desired cell of claim 21, or a descendant thereof, wherein  
31 said animal is either a chimeric or a transgenic animal.  
32

-76-

1        27. Th n n-human animal of claim 26, wherein said animal  
2 and said desir d cell are f the same sp ci s, and wherein  
3 said species is selected from the group consisting of: a  
4 chicken, a mouse, a rat, a hamster, a rabbit, a sheep, a  
5 goat, a fish, a pig, a cow or bull, and a non-human primate.  
6

7        28. A non-fungal plant containing a cell derived from the  
8 desired cell of claim 5, or a descendant thereof, wherein  
9 said non-fungal plant is either a chimeric or a transgenic  
10 plant.  
11

12        29. A method of gene therapy which comprises introducing  
13 to a recipient in need of such therapy, a desired non-  
14 selectable gene sequence, said method comprising:

15        A. providing to said recipient an effective amount  
16 of a DNA molecule containing said desired non-selectable  
17 gene sequence, wherein said DNA molecule additionally  
18 contains two regions of homology which flank said desired  
19 gene sequence, and which are sufficient to permit said  
20 desired gene sequence to undergo homologous recombination  
21 with a predetermined gene sequence present in a precursor  
22 cell of said recipient;

23        B. permitting said DNA molecule to be introduced  
24 into said precursor cell;

25        C. permitting said introduced DNA molecule to  
26 undergo homologous recombination with said predetermined  
27 gene sequence of said genome of said precursor cell to  
28 thereby produce a desired cell wherein said desired non-  
29 selectable gene sequence has been inserted into said  
30 predetermined gene sequence; and wherein the presence or  
31 expression of said introduced gene sequence in said cell of  
32 said recipient comprises said gene therapy.  
33

1           30. The method of claim 29 wherein said recipient is a  
2 non-fungal plant.

3           31. The method of claim 29 wherein said recipient is an  
4 animal.  
5

6           32. The method of claim 31 wherein said animal is  
7 selected from the group consisting of: a chicken, a mouse,  
8 a rat, a hamster, a rabbit, a sheep, a goat, a fish, a pig,  
9 a cow or bull, a non-human primate and a human.  
10

11           33. The method of claim 32, wherein said animal is a  
12 human.  
13

14           34. A method for obtaining a desired animal or non-fungal  
15 plant cell which contains a desired non-selectable gene  
16 sequence inserted within a predetermined gene sequence of  
17 said cell's genome, which method comprises:  
18

19           A. incubating a precursor cell under non-selective  
20 culture conditions, or under a first set of selective  
21 culture conditions, with a DNA molecule containing:

22           i) said desired non-selectable gene sequence,  
23 wherein said DNA molecule additionally contains  
24 two regions of homology which flank said desired  
25 gene sequence, and which are sufficient to permit  
26 said desired gene sequence to undergo homologous  
27 recombination with said predetermined gene  
28 sequence of said genome of said precursor cell;  
29 and

30           ii) a selectable gene sequence whose presence or  
31 expression in said precursor cell can be selected  
32 for by culturing said cell under said first set  
33 of selective culture conditions, and whose

1                   presence or expression in said precursor cell can  
2                   be selected against by culturing said cell under  
3                   a second set of selective culture conditions;

4                   B. permitting said DNA molecule to be introduced  
5                   into said precursor cell;

6                   C. permitting said introduced DNA molecule to  
7                   undergo homologous recombination with said predetermined  
8                   gene sequence of said genome of said precursor cell to  
9                   thereby produce said desired cell wherein said desired non-  
10                  selectable gene sequence has been inserted into said  
11                  predetermined gene sequence; and

12                  D. recovering said desired cell by culturing said  
13                  cell under said first set of selective culture conditions,  
14                  by then permitting said cell to undergo intrachromosomal  
15                  recombination under non-selective culture conditions, and by  
16                  then incubating said cell under said second set of selective  
17                  culture conditions.

18  
19                  35. The method of claim 34, wherein said cell is  
20                  deficient in HPRT enzyme, and wherein said selectable gene  
21                  sequence expresses an active HPRT enzyme, and wherein said  
22                  first set of selective culture conditions comprises  
23                  incubation of said cell under conditions in which the  
24                  presence of an active HPRT enzyme in said cell is required  
25                  for growth, and wherein said second set of selective culture  
26                  conditions comprises incubation of said cell under  
27                  conditions in which the absence of an active HPRT enzyme in  
28                  said cell is required for growth.

29  
30                  36. The method of claim 34, wherein said cell is  
31                  deficient in APRT enzyme, and wherein said selectable gene  
32                  sequence expresses an active APRT enzyme, and wherein said  
33                  first set of selective culture conditions comprises

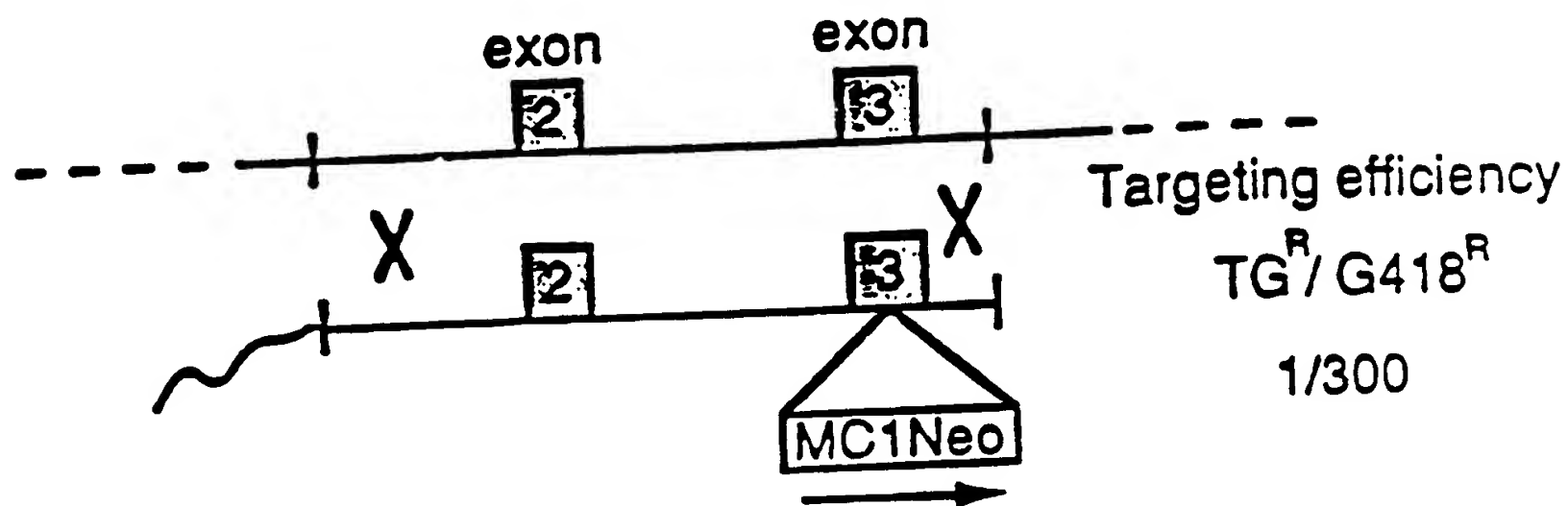


1 incubation of said cell under conditions in which the  
2 presence of an active APRT enzyme in said cell is required  
3 for growth, and wherein said second set of selective culture  
4 conditions comprises incubation of said cell under  
5 conditions in which the absence of an active APRT enzyme in  
6 said cell is required for growth.

7  
8 37. The method of claim 34, wherein said cell is  
9 deficient in TK enzyme, and wherein said selectable gene  
10 sequence expresses an active TK enzyme, and wherein said  
11 first set of selective culture conditions comprises  
12 incubation of said cell under conditions in which the  
13 presence of an active TK enzyme in said cell is required for  
14 growth, and wherein said second set of selective culture  
15 conditions comprises incubation of said cell under  
16 conditions in which the absence of an active TK enzyme in  
17 said cell is required for growth.

- 1 / 17

1A Replacement vectors: 6.8kb Homology which target the *hprt* locus



1B Insertion Vectors : 6.8kb homology

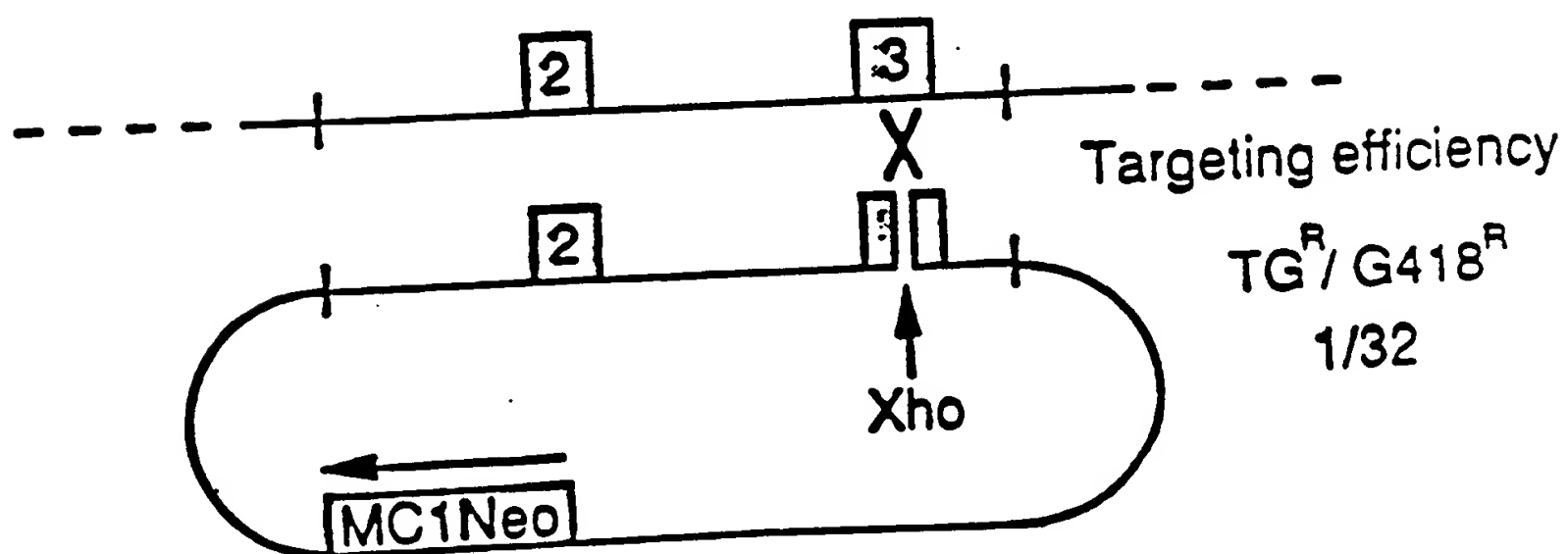
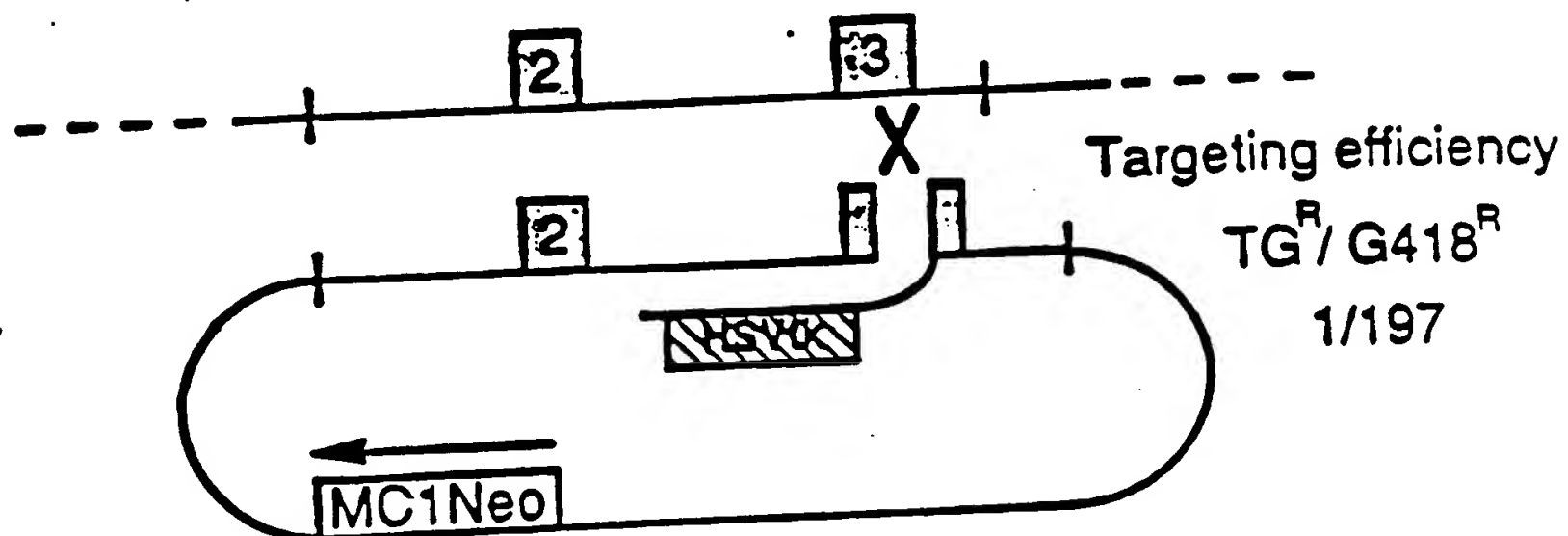


FIGURE 1

- 2 / 17

A. Insertion vectors with heterology at the insertion site: 2kb  
insertion at the linearization site



2B Insertion vectors with heterology at the insertion site: 26bp  
insertion at the linearization site

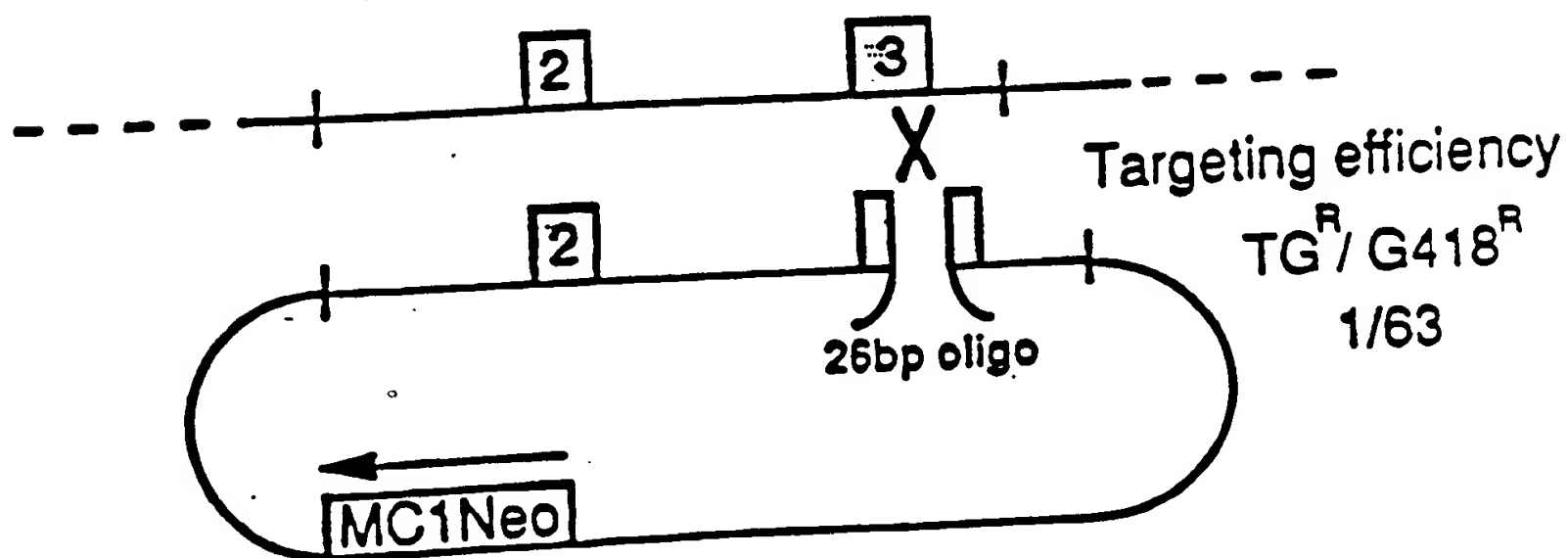


FIGURE 2

- 3 / 17

2c Insertion vectors with added heterology: Internal to the homology

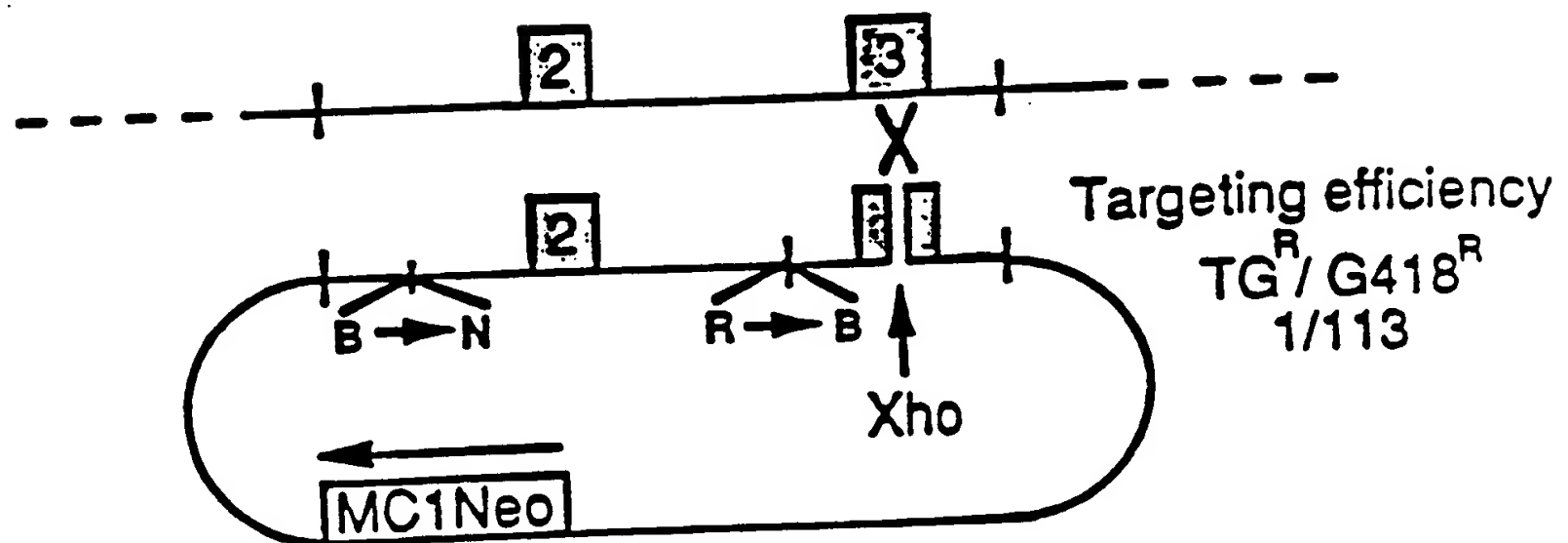
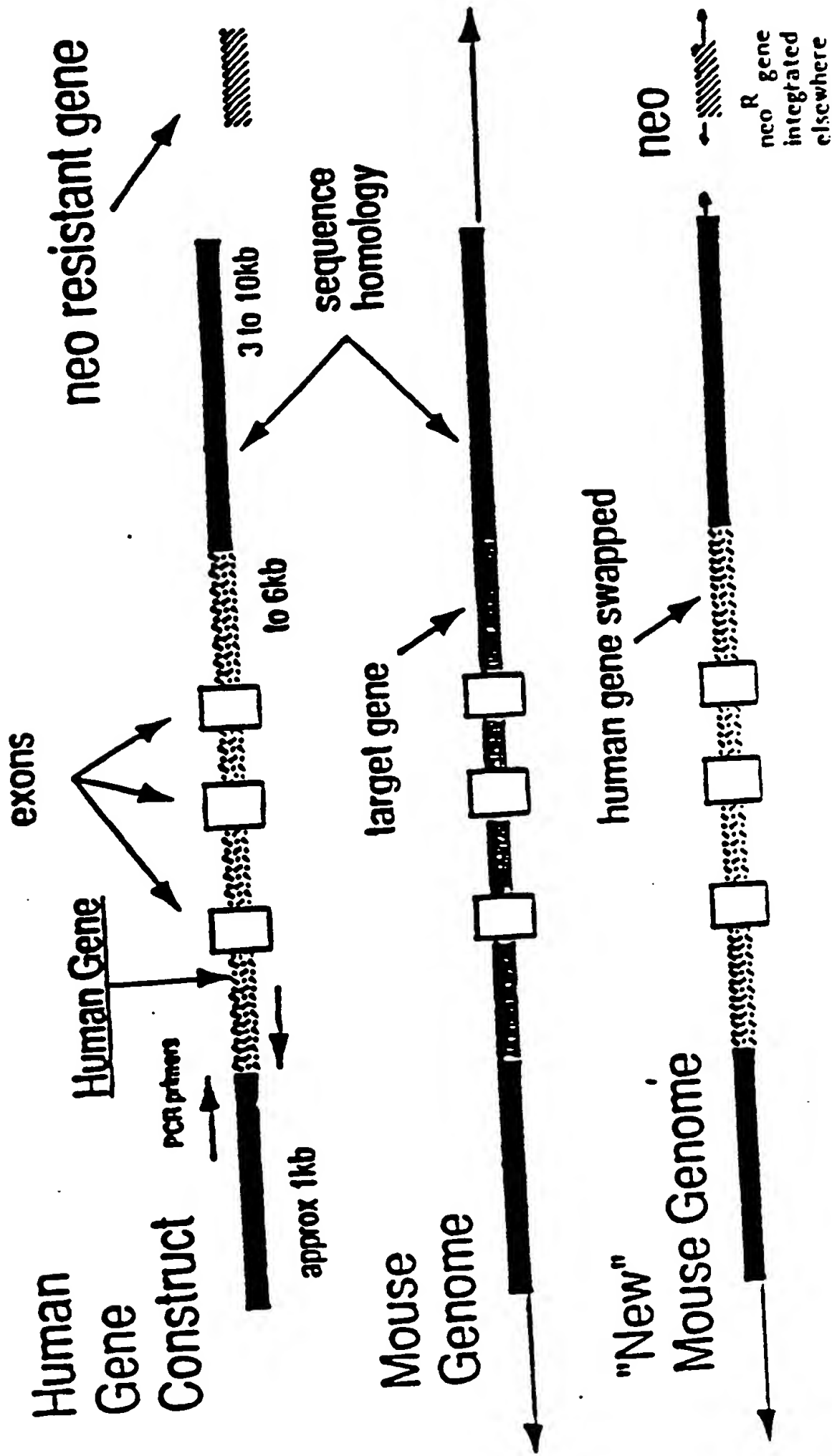


FIGURE 3  
Genetic Replacement Technology



- 5 / 17

A Step#1, homologous recombination: Adding the human replacement with an insertion vector.

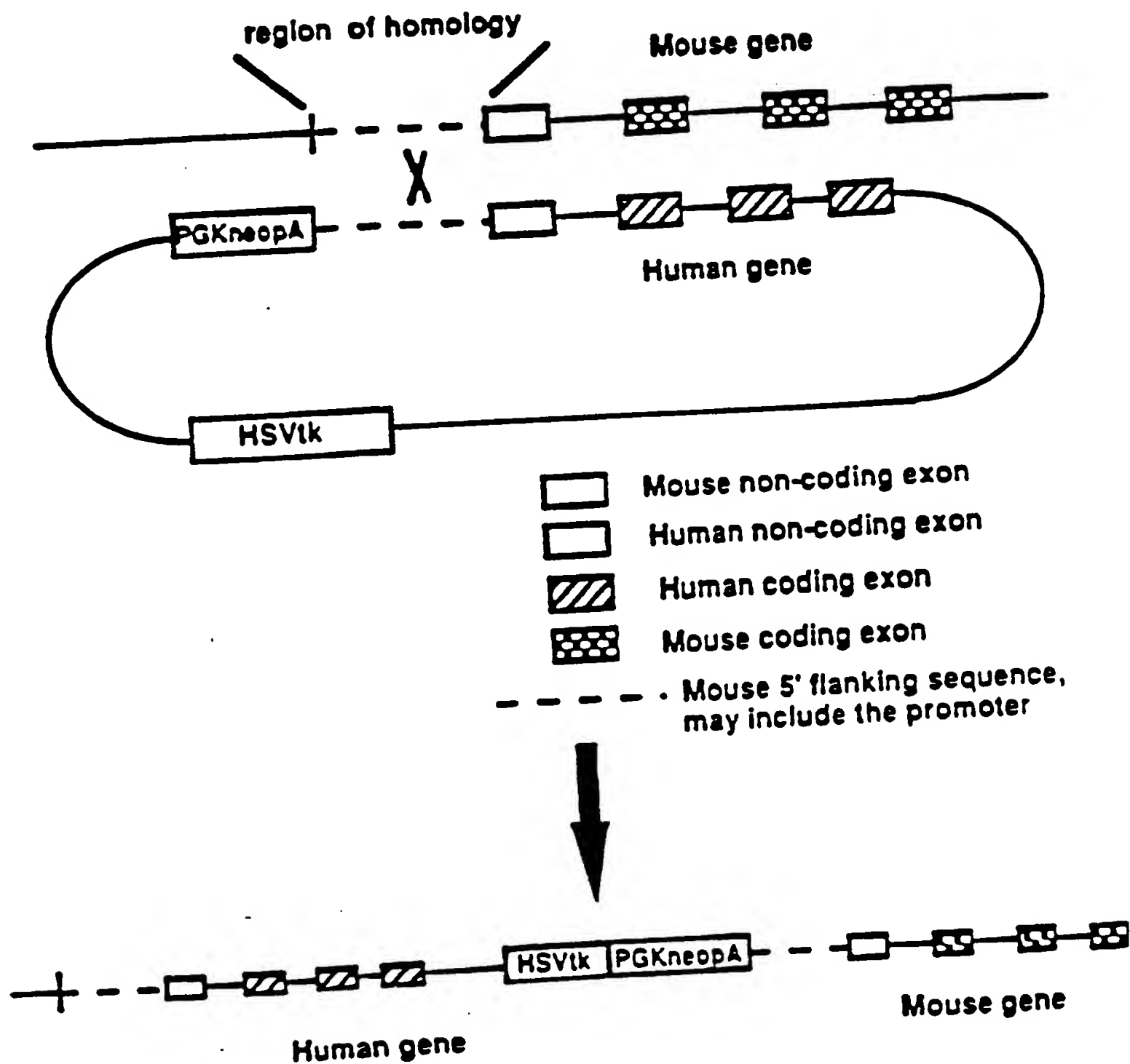


FIGURE 4

- 6 / 17

Step#2: Reconstruct junction, remove duplicated promoter, add additional 3' human sequences. Select in - FIAU (100%)

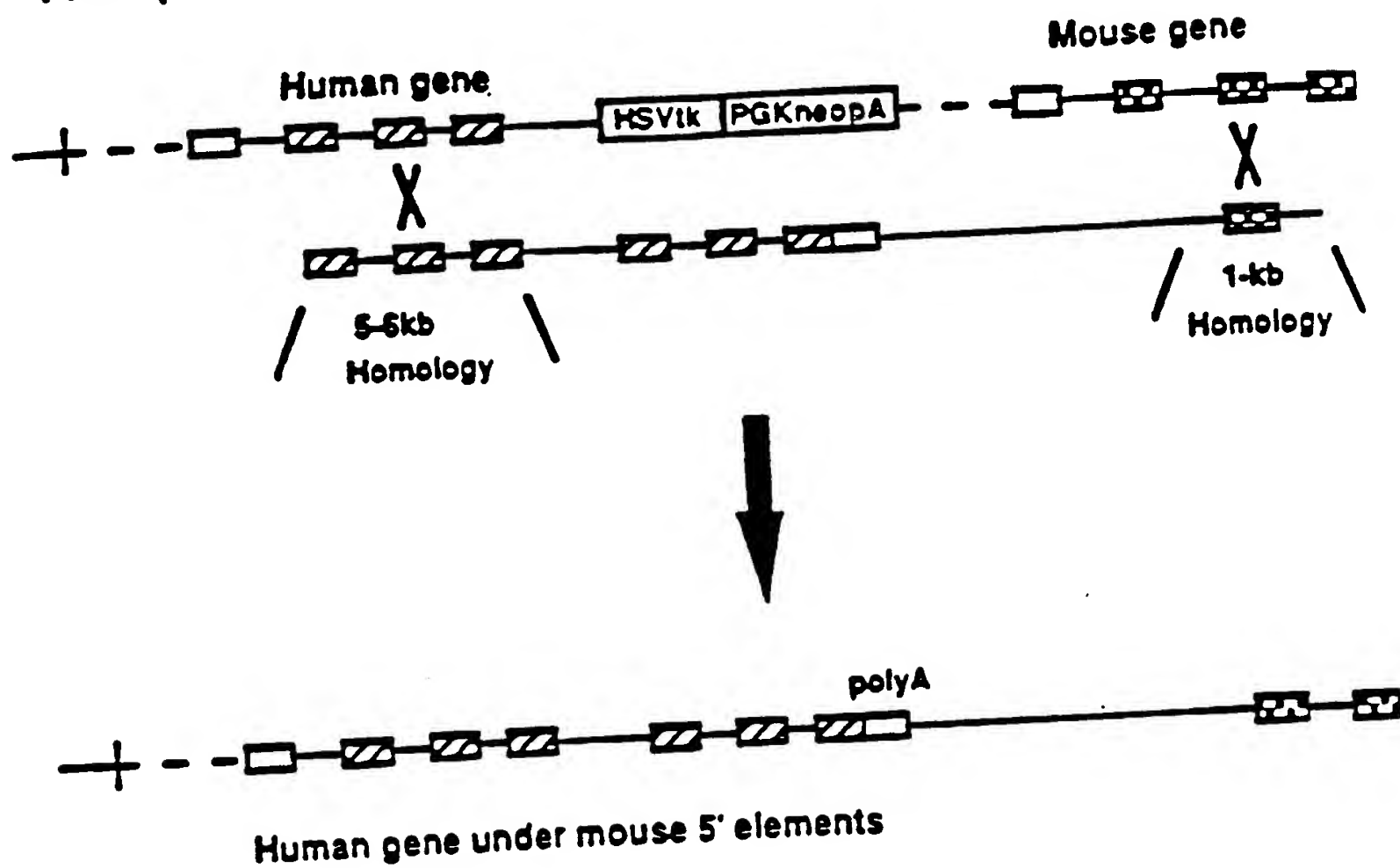
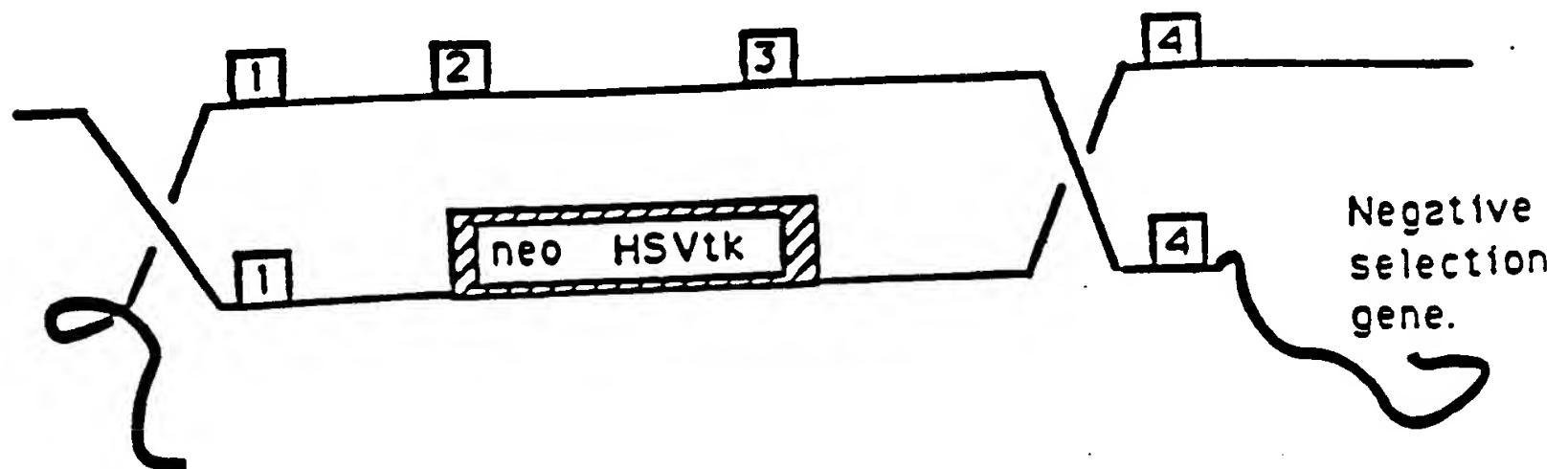


FIGURE 4 (continued)

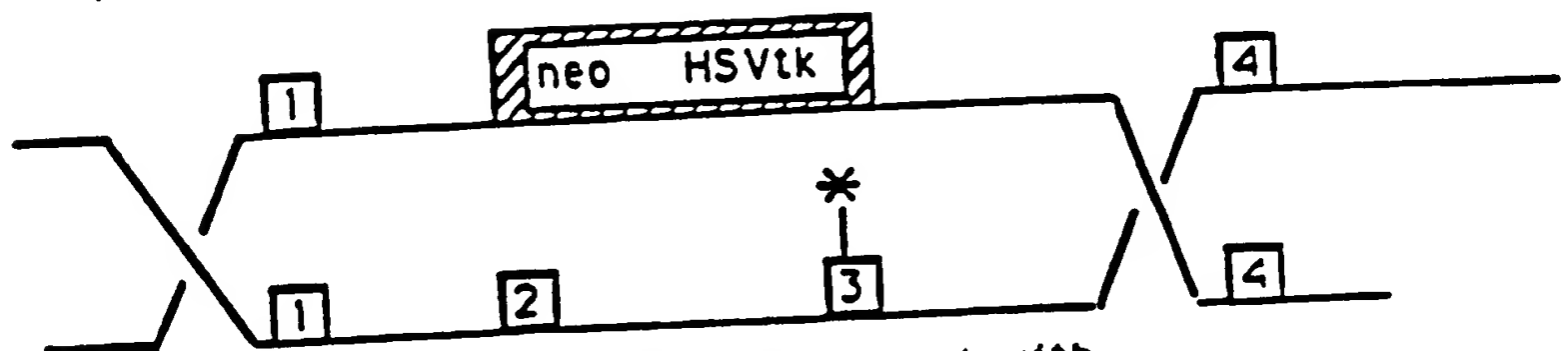
- 7 / 17

# Selective introduction of small and large genomic changes

Step<sup>1</sup> - Standard Replacement vector



Step<sup>2</sup> A - Introduction of small mutations



Step<sup>2</sup> B - complete or partial replacement with homologous or heterologous sequences.

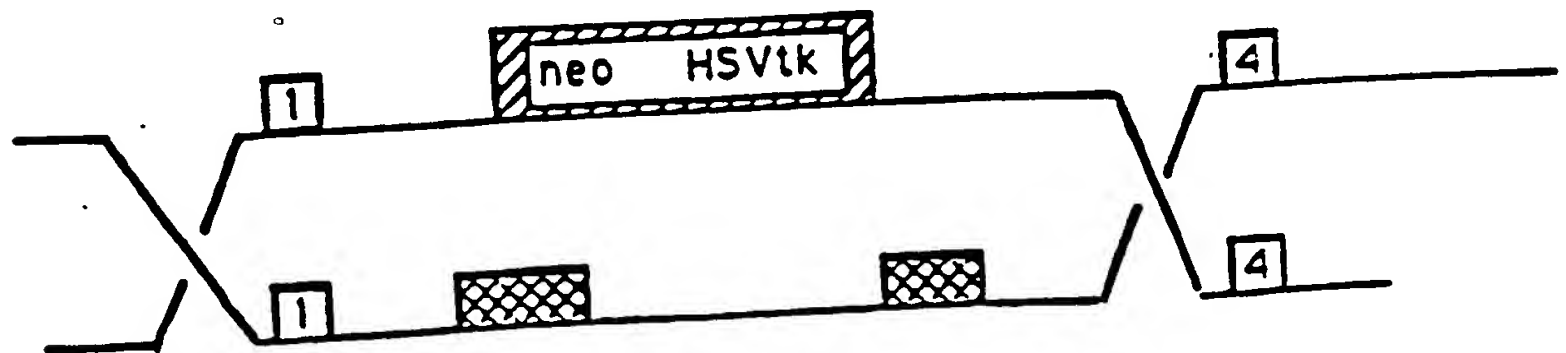
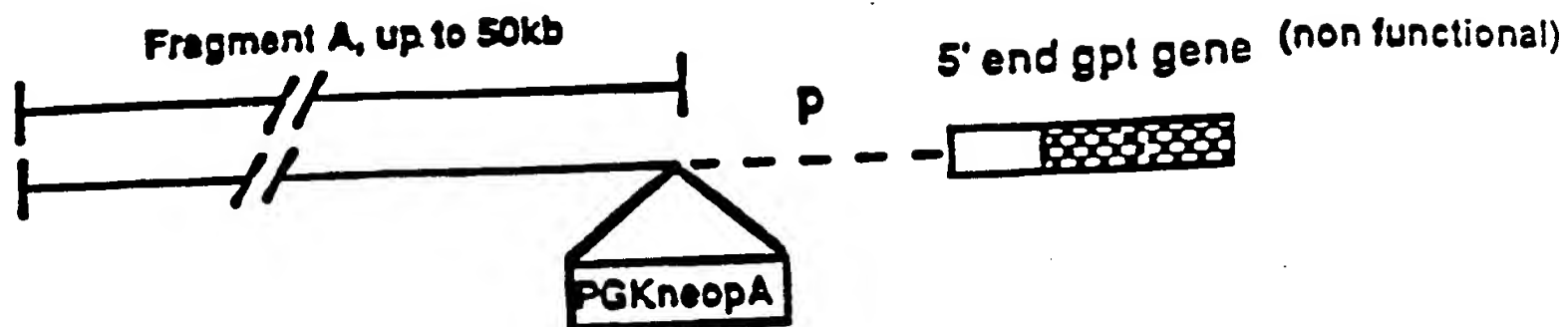


FIGURE 5



[- 8 / 17

6A

**Step #1 Set up the initial target**

6B

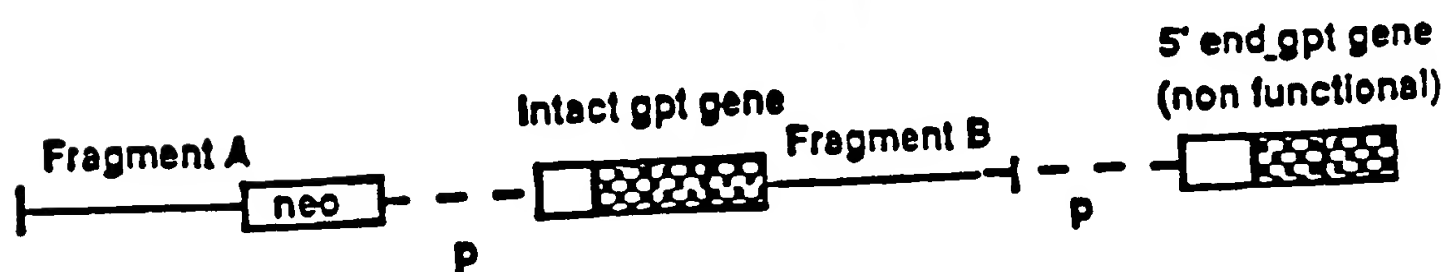
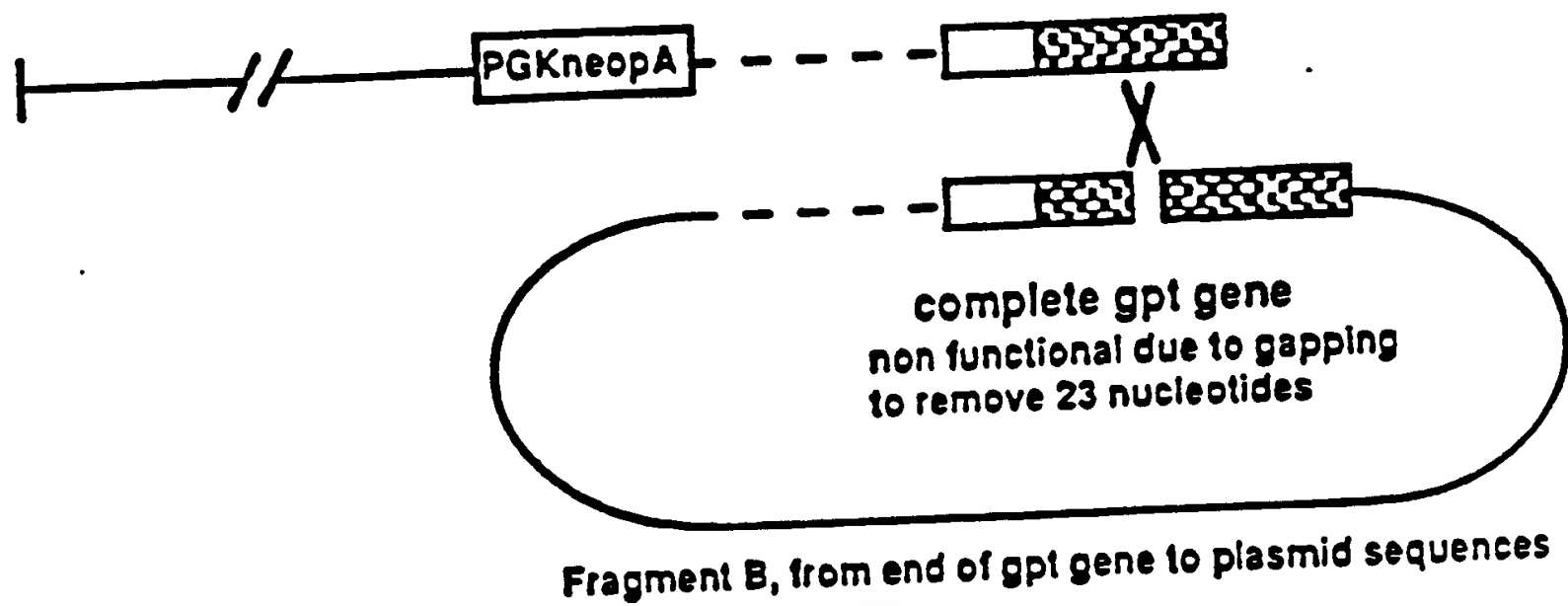
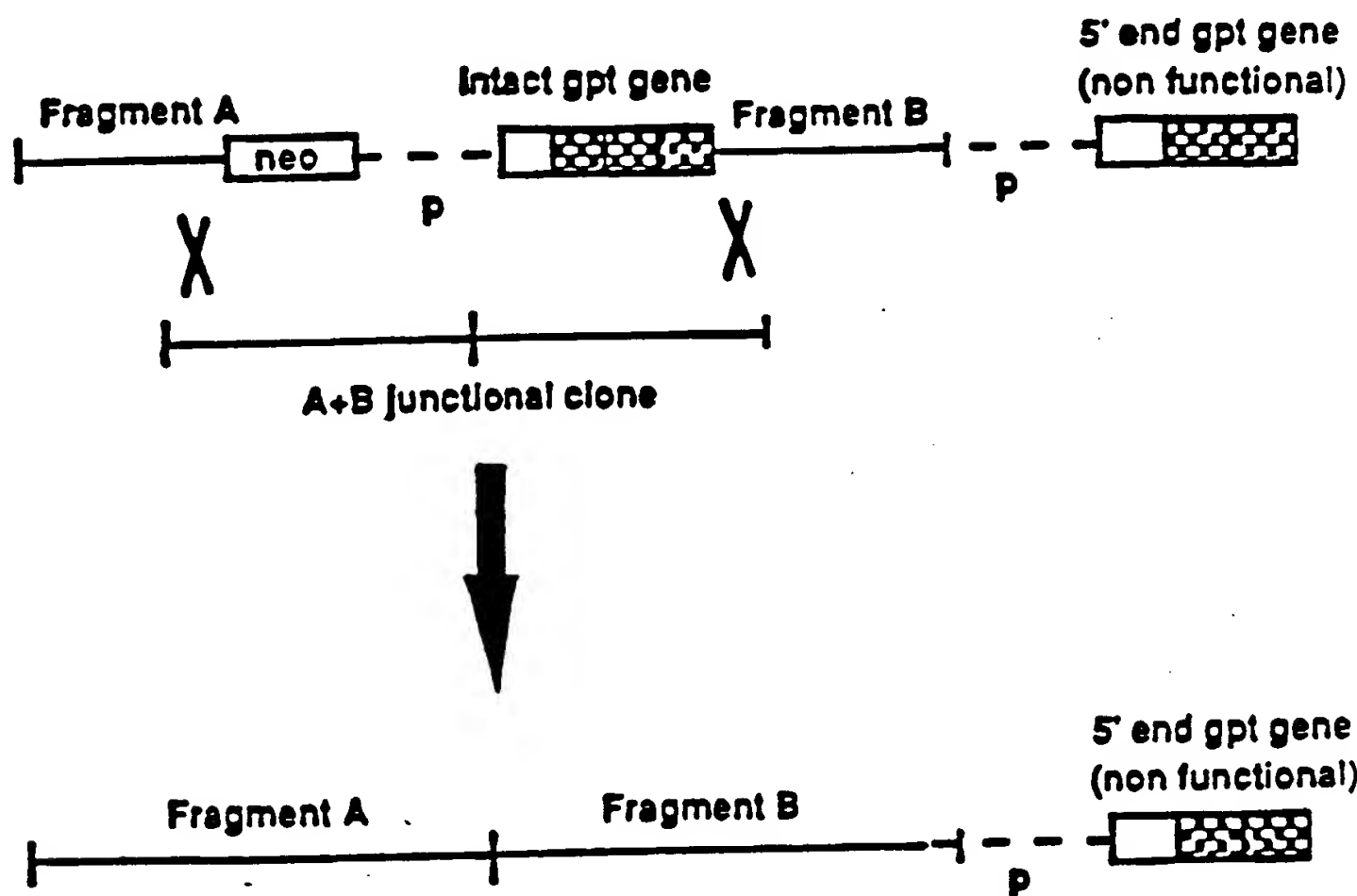
**Step#2, homologous recombination: Adding the contiguous clone: Selection in HAT**

FIGURE 6

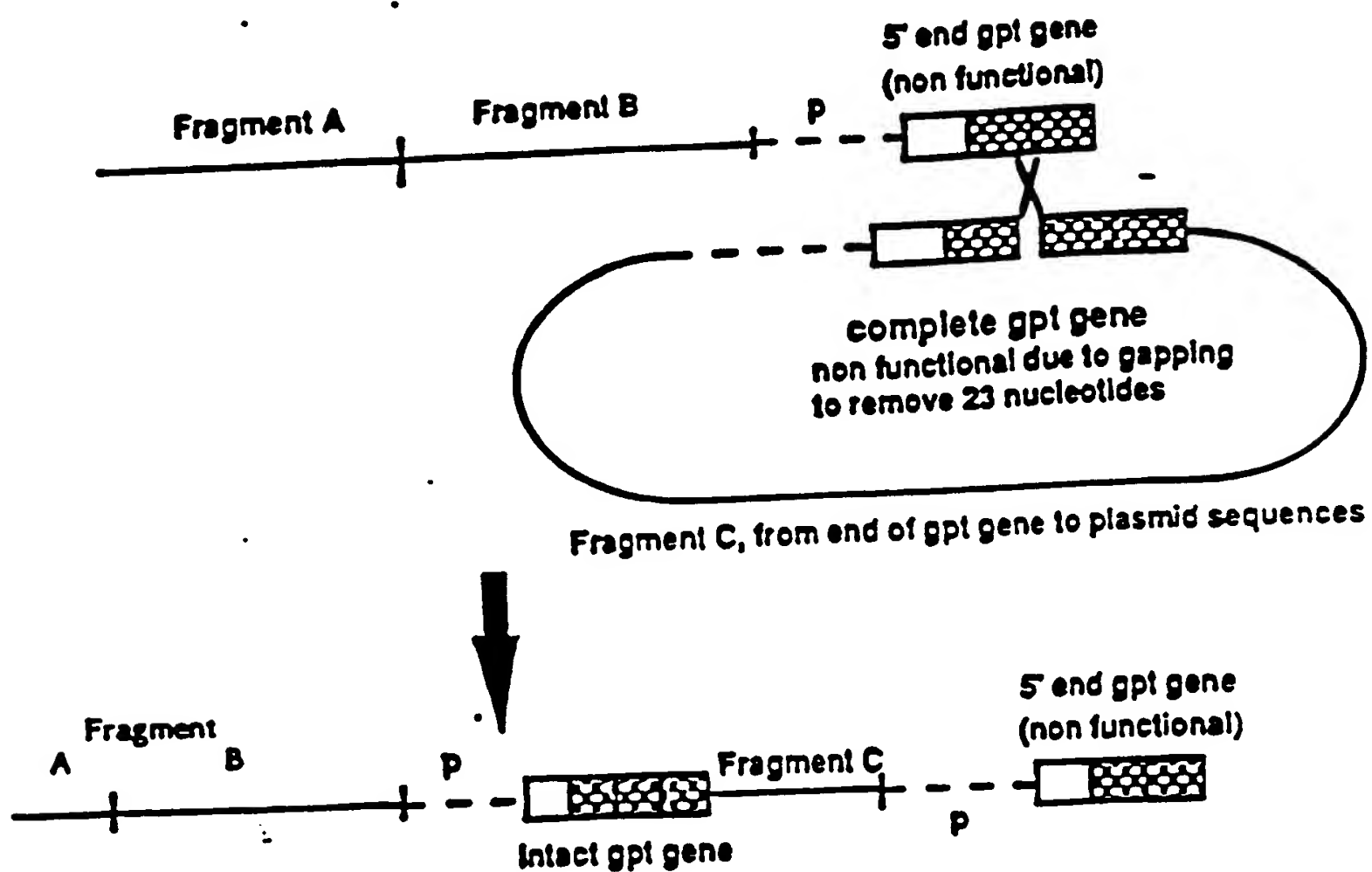
- 9 / 17

<sup>6C</sup>  
**Step#3: Repair A-B junction. Select in - 6TG (100%)**

**FIGURE 6 (continued)**

6D

## Step #4: Addition of contiguous clone



6E

## Step#5: Repair with B+C junctional clone. Select in - 6TG (100%)

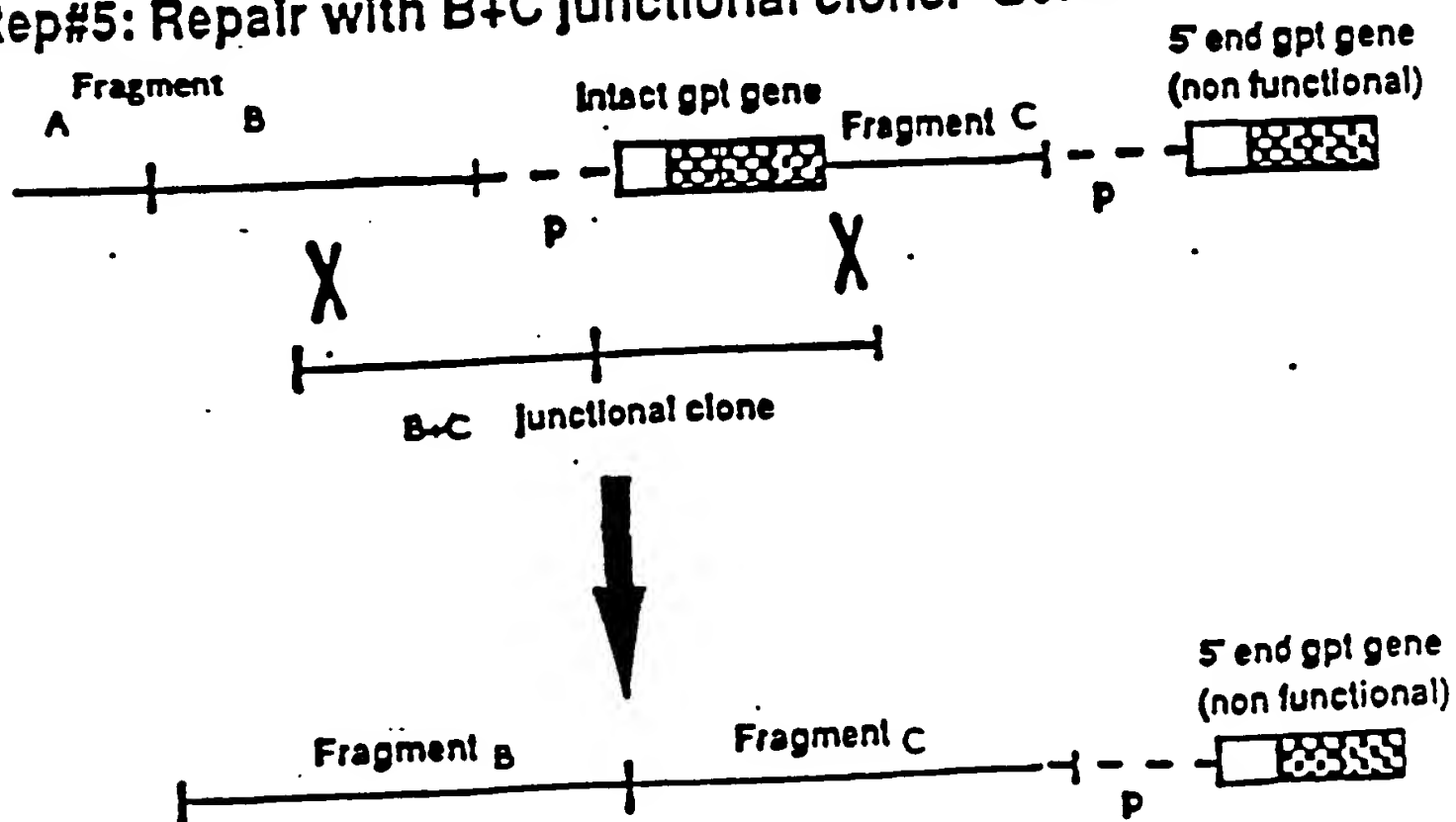


FIGURE 6 (continued)

11/17

## Co-Electroporation

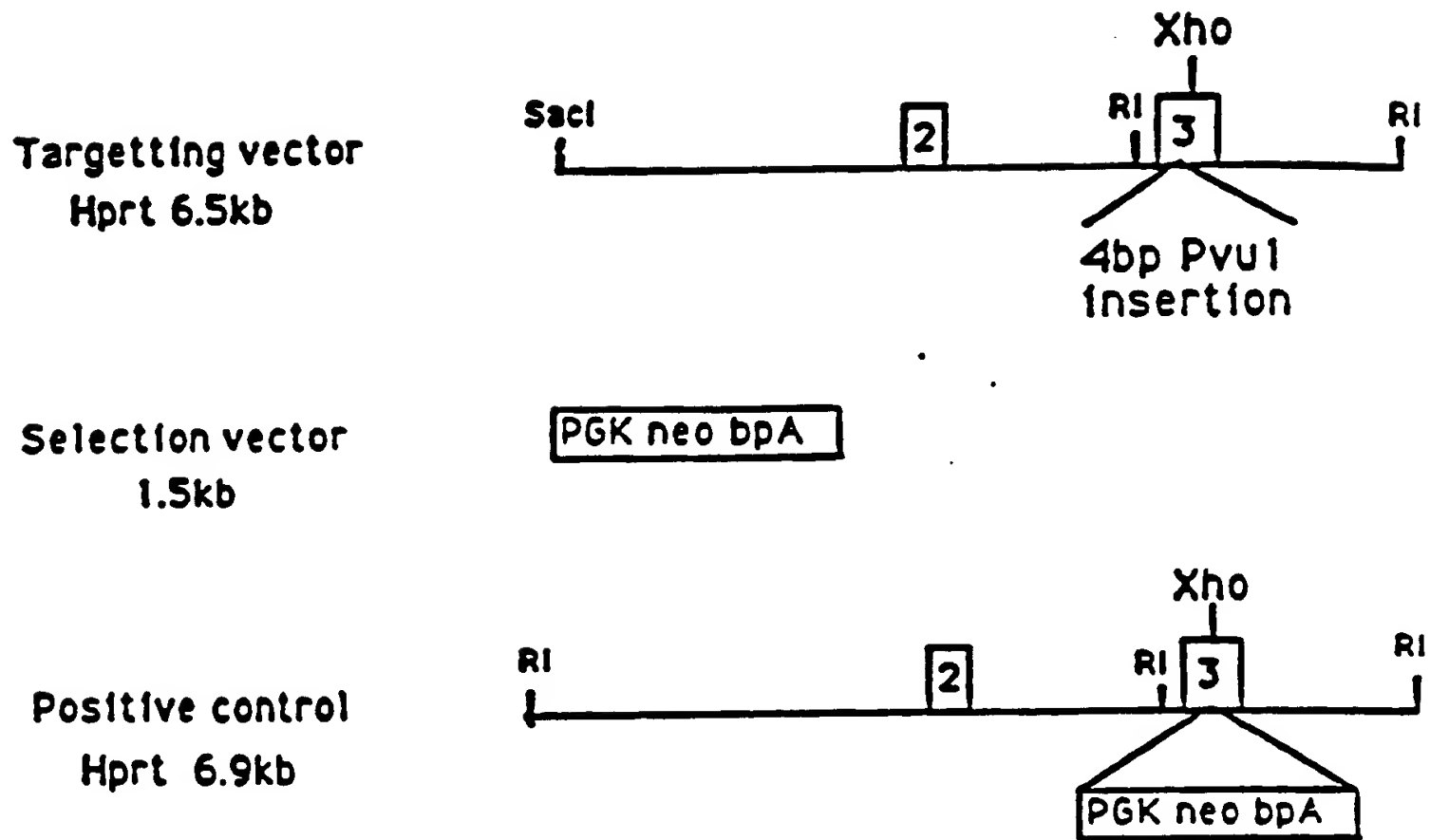


FIGURE 7

12/17

Predicted structure of the *hprt* locus following homologous integration of the IV6.8 vector

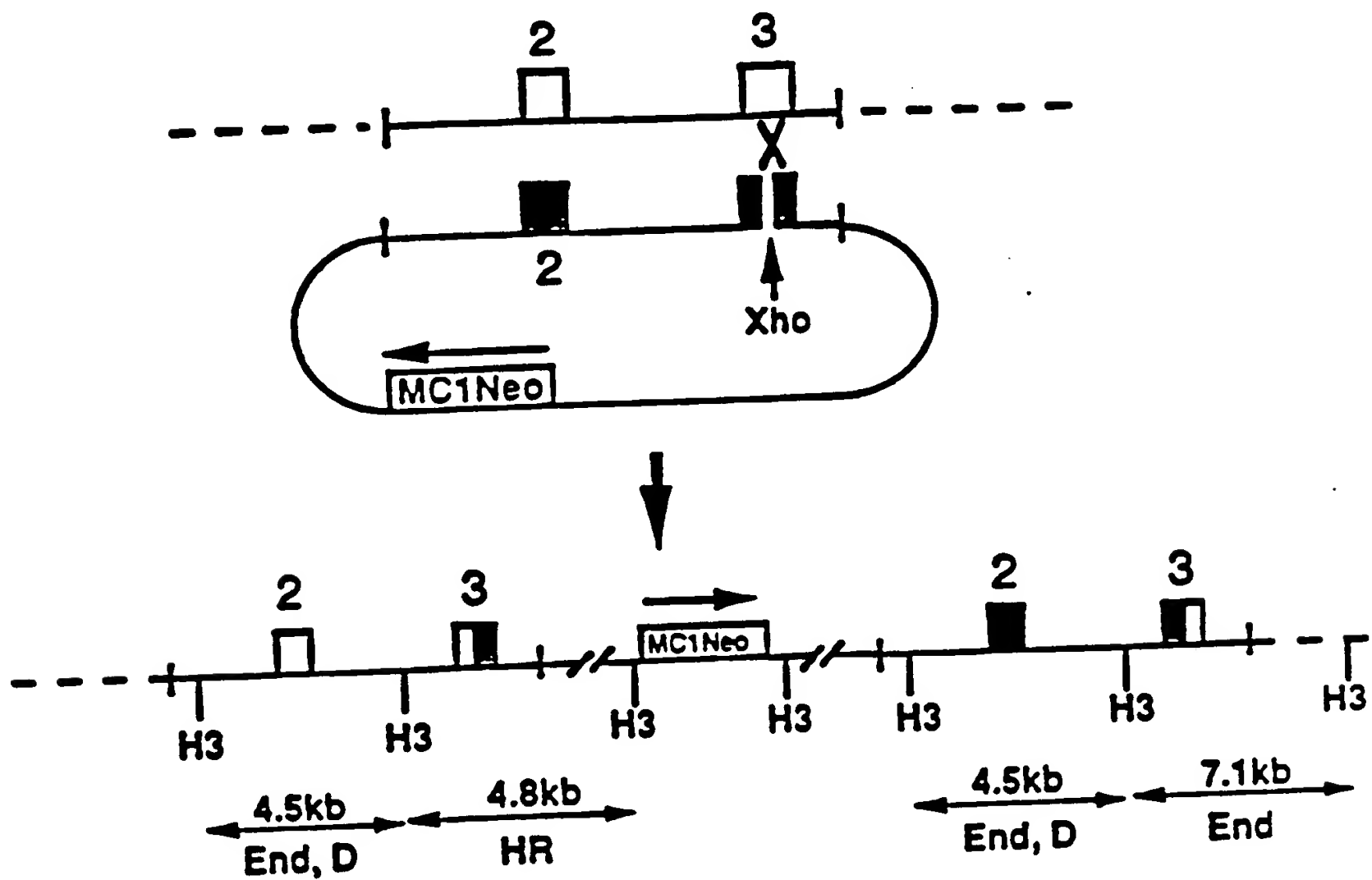


FIGURE 8

13 / 17

# Reversion of homologous recombinants generated with insertion vectors

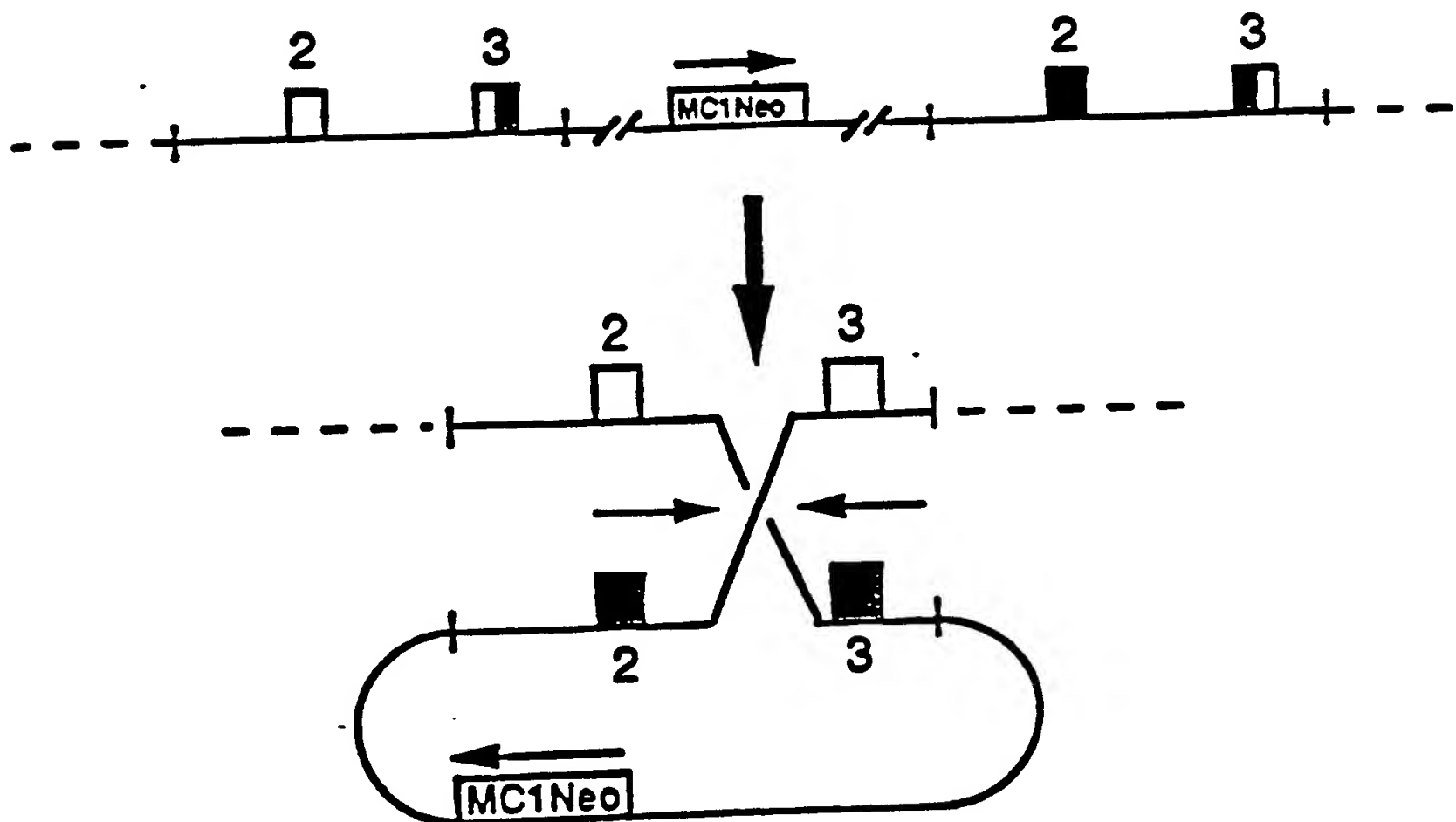


FIGURE 9

14 / 17

## Poly A selection

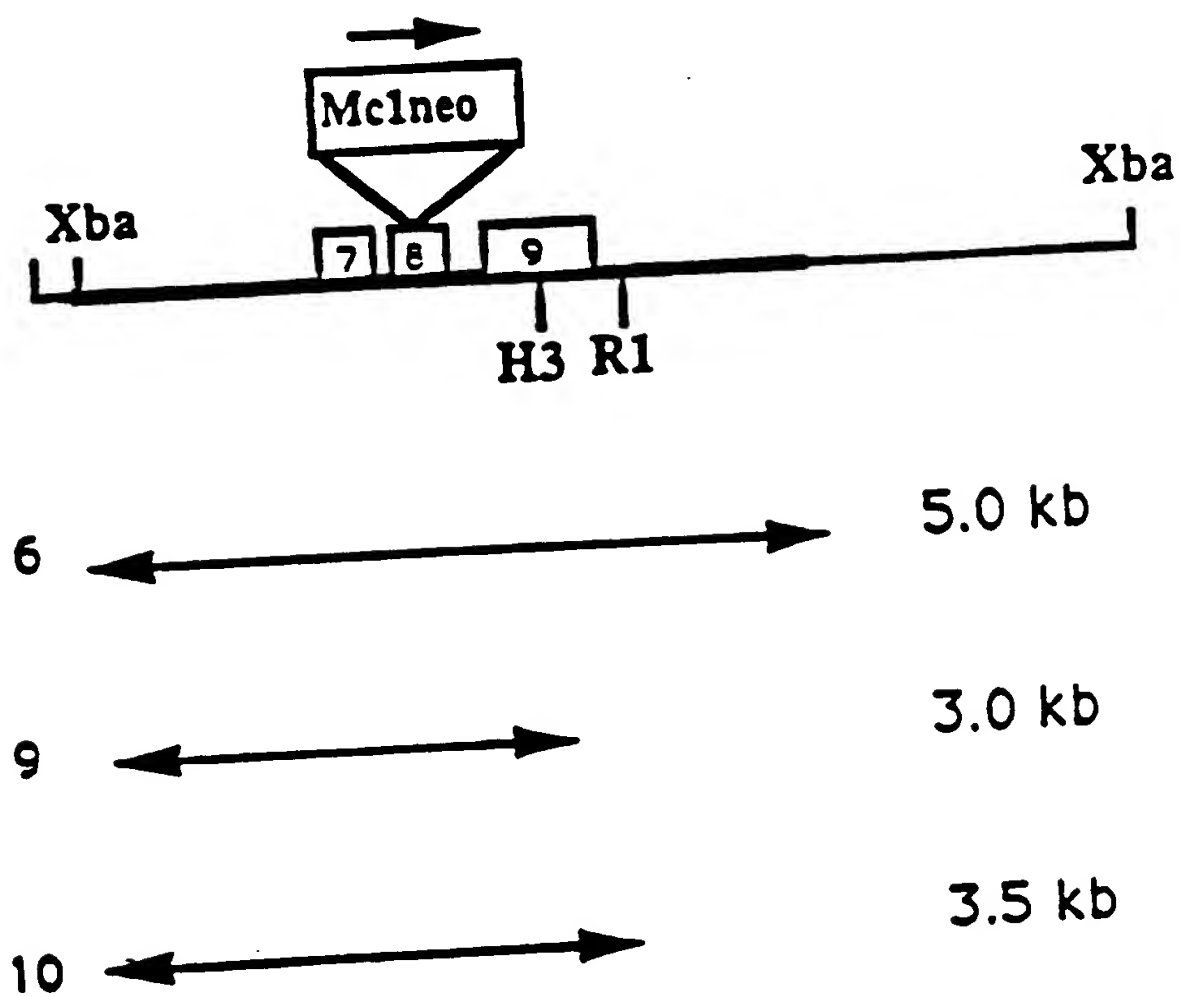
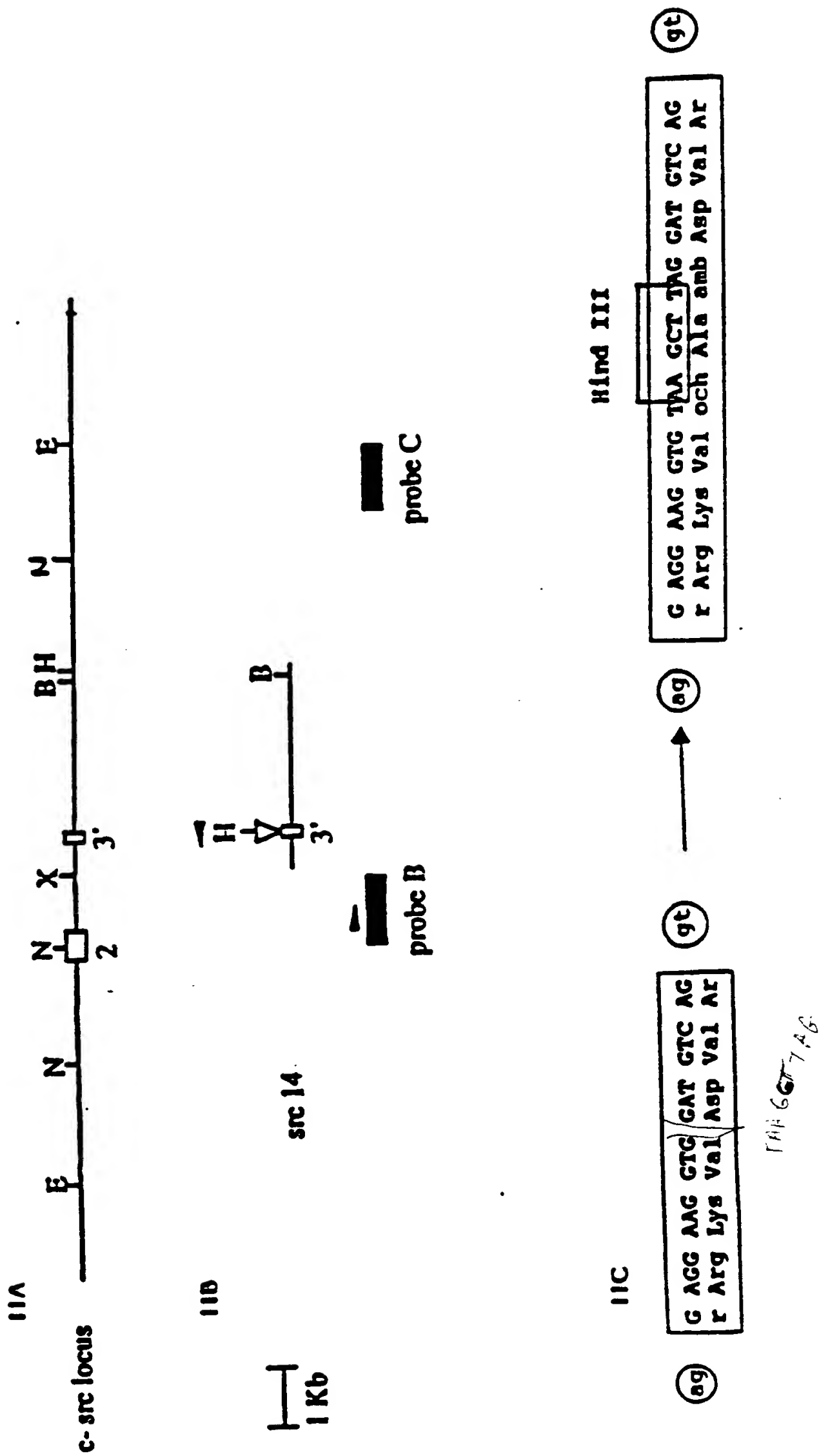


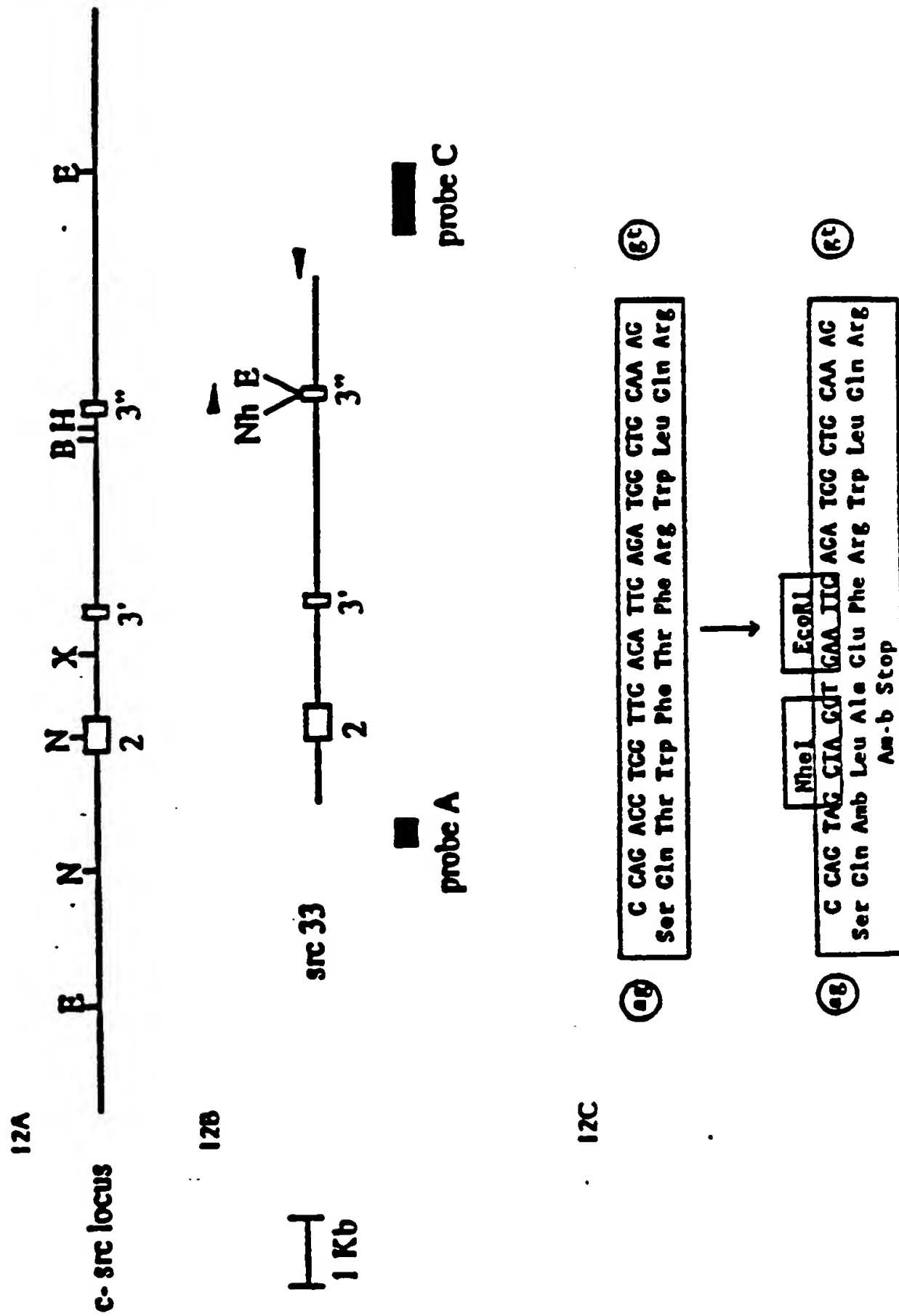
FIGURE 10

FIGURE 11





## FIGURE 12



17 / 17

# Direct selection for targeted recombination events

**Targeted**

**Random**

Single copy or concatameric integrations

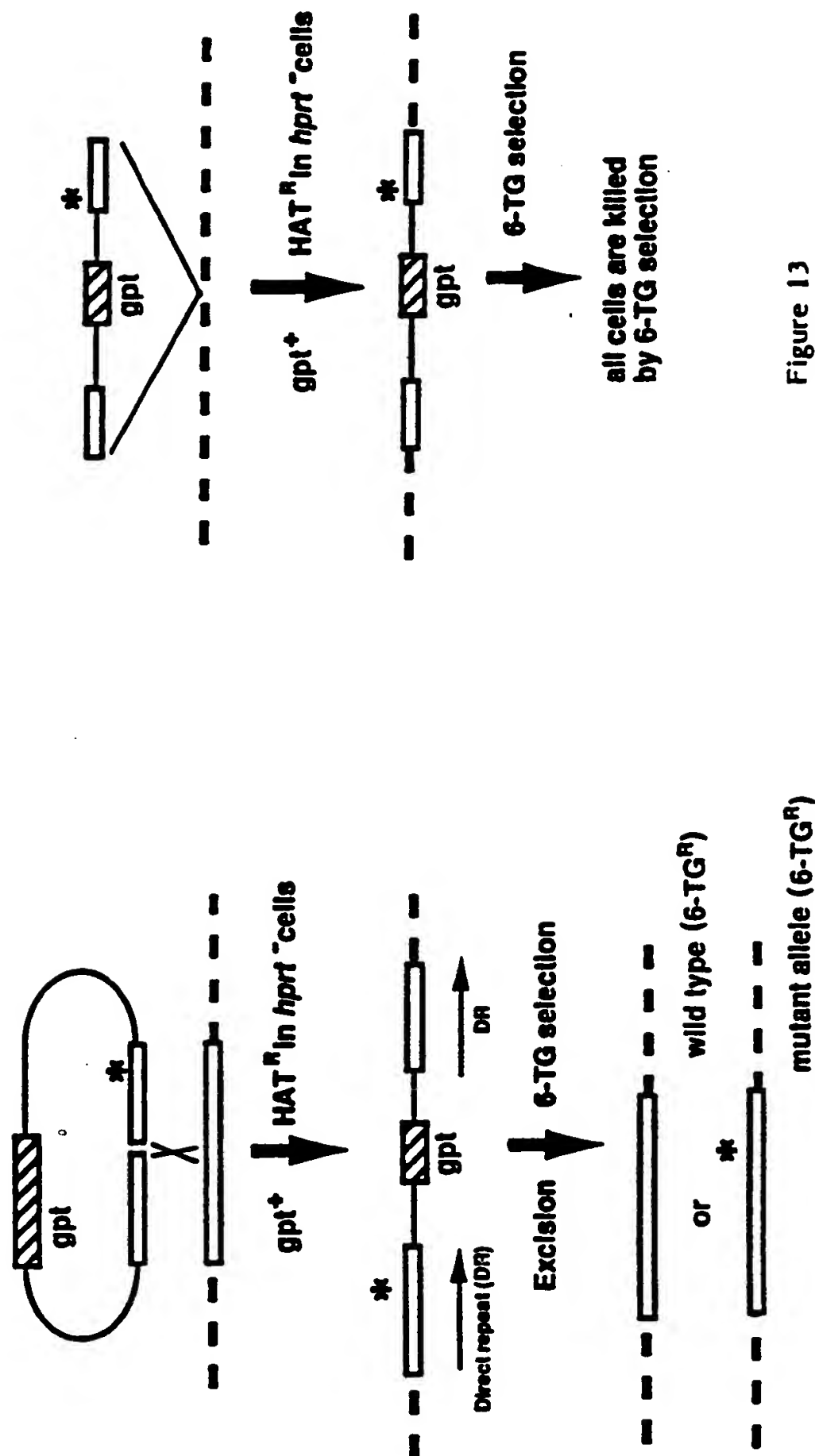


Figure 13

# INTERNATIONAL SEARCH REPORT

International Application No. **PCT/US91/04006**

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) * According to International Patent Classification (IPC) or to both National Classification and IPC <b>I.P.C.(5):</b> <u>C12N 15/00, 5/00</u> <b>U.S.CI:</b> <u>435/172.3, 240.1; 935/55, 56, 70</u>														
<b>II. FIELDS SEARCHED</b> <div style="text-align: right; font-size: small;">Minimum Documentation Searched ?</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 25%; border-bottom: 1px solid black;">Classification System</th> <th style="border-bottom: 1px solid black;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: middle;">U.S.</td> <td style="border: 1px solid black;">435/172.3, 240.1; 935/55, 56 70</td> </tr> </table> <div style="text-align: center; font-size: x-small; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *</div>			Classification System	Classification Symbols	U.S.	435/172.3, 240.1; 935/55, 56 70								
Classification System	Classification Symbols													
U.S.	435/172.3, 240.1; 935/55, 56 70													
<b>APS, Chemical Abstracts, Biological Abstracts</b>														
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 10%; border-bottom: 1px solid black;">Category *</th> <th style="width: 70%; border-bottom: 1px solid black;">Citation of Document, ** with indication, where appropriate, of the relevant passages **</th> <th style="width: 20%; border-bottom: 1px solid black;">Relevant to Claim No. 13</th> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">X Y</td> <td style="border: 1px solid black; vertical-align: top;">           Proceedings of the National Academy of Science, vol. 85, issued November 1988, Doetschman et al, "Targeted mutation of the <u>Hrpt</u> Gene in Mouse Embryonic Stem Cells," pages 8583-8587, see entire document.         </td> <td style="border: 1px solid black; vertical-align: top; text-align: center;">           1-4, 6-16            18-21, 34            35-37         </td> </tr> <tr> <td style="border: 1px solid black; text-align: center; vertical-align: top;">X Y</td> <td style="border: 1px solid black; vertical-align: top;">           Science, Vol. 245, issued 15 September 1989, Johnson et al, "Targeting of Non expressed Genes in Embryonic Stem Cells via Homologous Recombination, pages 1234-1236, see entire document.         </td> <td style="border: 1px solid black; vertical-align: top; text-align: center;">           1-4, 6-16            18-21,            34-37         </td> </tr> <tr> <td colspan="3" style="border: 1px solid black; text-align: center; padding: 20px;">           (Next)         </td> </tr> </table>			Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. 13	X Y	Proceedings of the National Academy of Science, vol. 85, issued November 1988, Doetschman et al, "Targeted mutation of the <u>Hrpt</u> Gene in Mouse Embryonic Stem Cells," pages 8583-8587, see entire document.	1-4, 6-16 18-21, 34 35-37	X Y	Science, Vol. 245, issued 15 September 1989, Johnson et al, "Targeting of Non expressed Genes in Embryonic Stem Cells via Homologous Recombination, pages 1234-1236, see entire document.	1-4, 6-16 18-21, 34-37	(Next)		
Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages **	Relevant to Claim No. 13												
X Y	Proceedings of the National Academy of Science, vol. 85, issued November 1988, Doetschman et al, "Targeted mutation of the <u>Hrpt</u> Gene in Mouse Embryonic Stem Cells," pages 8583-8587, see entire document.	1-4, 6-16 18-21, 34 35-37												
X Y	Science, Vol. 245, issued 15 September 1989, Johnson et al, "Targeting of Non expressed Genes in Embryonic Stem Cells via Homologous Recombination, pages 1234-1236, see entire document.	1-4, 6-16 18-21, 34-37												
(Next)														
<div style="display: flex; justify-content: space-between; font-size: x-small;"> <div style="width: 45%;"> <p>* Special categories of cited documents: **</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"G" document member of the same patent family</p> </div> </div>														
<b>IV. CERTIFICATE</b> <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: bottom;">           Date of the Actual Completion of the International Search   <b>28 August 1991</b> </td> <td style="width: 50%; border-bottom: 1px solid black; vertical-align: bottom;">           Date of Mailing of this International Search Report   <b>24 SEP 1991</b> </td> </tr> <tr> <td style="border-bottom: 1px solid black; vertical-align: bottom;">           International Searching Authority   <b>ISA/US</b> </td> <td style="border-bottom: 1px solid black; vertical-align: bottom;">           Signature of Authorized Official  <b>Deborah Couch</b> </td> </tr> </table>			Date of the Actual Completion of the International Search  <b>28 August 1991</b>	Date of Mailing of this International Search Report  <b>24 SEP 1991</b>	International Searching Authority  <b>ISA/US</b>	Signature of Authorized Official <b>Deborah Couch</b>								
Date of the Actual Completion of the International Search  <b>28 August 1991</b>	Date of Mailing of this International Search Report  <b>24 SEP 1991</b>													
International Searching Authority  <b>ISA/US</b>	Signature of Authorized Official <b>Deborah Couch</b>													

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
YES	Molecular and Cellular Biology, Vol. 10, issued February 1990, Raur et al, "Intermolecular Recombination in Plants," 492-500, see entire document.	1-5, 12, 17 19, 21 34-37
YES	The Plant Cell, Vol. 2, issued May 1990, "Homologous Recombination in Plant Cells after <u>Agrobacterium</u> Mediated Transformation," pages 415-425, see entire document.	1-5, 12, 17 19, 21 34-37

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

$\frac{N}{Y}$	Nature, Volume 338, issued 09 March 1989, Joyner et al, "Production of a mutation in Mouse <u>Fu-2</u> Gene by Homologous Recombination in Embryonic Stem Cells," pages 153-156, see entire document.	$\frac{1-4, 6-16}{18-21.}$ $\frac{34-37}{}$
---------------	---	--

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ..... because they relate to subject matter is not required to be searched by this Authority, namely:

2. ☐ Claim numbers ..... because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out is, specifically:

3. ☐ Claim numbers ..... because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 8A(2).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This international Searching Authority found multiple inventions in this international application as follows:

**See Attachment**

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

**1-21 and 34-37 telephone practice**

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.